THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 13, Issue of March 30, pp. 10083–10096, 2001

Printed in U.S.A.

Distinct Arachidonate-releasing Functions of Mammalian Secreted Phospholipase A_2s in Human Embryonic Kidney 293 and Rat Mastocytoma RBL-2H3 Cells through Heparan Sulfate Shuttling and External Plasma Membrane Mechanisms*

Received for publication, August 29, 2000, and in revised form, October 6, 2000
Published, JBC Papers in Press, December 5, 2000, DOI 10.1074/jbc.M007877200

Makoto Murakami‡, Rao S. Koduri§, Ayako Enomoto‡, Satoko Shimbara‡, Mimie Seki‡, Kumiko Yoshihara‡, Alan Singer§, Emmanuel Valentin¶, Farideh Ghomashchi§, Gerard Lambeau¶, Michael H. Gelb§, and Ichiro Kudo‡‖

From the ‡Department of Health Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan, the §Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195-1700, and the ¶Institut de Pharmacologie Moleculaire et Cellulaire, CNRS-UPR 411, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

We analyzed the ability of a diverse set of mammalian secreted phospholipase A_2 (sPLA$_2$) to release arachidonate for lipid mediator generation in two transfected cell lines. In human embryonic kidney 293 cells, the heparin-binding enzymes sPLA$_2$-IIA, -IID, and -V promote stimulus-dependent arachidonic acid release and prostaglandin E_2 production in a manner dependent on the heparan sulfate proteoglycan glypican. In contrast, sPLA$_2$-IB, -IIC, and -IIE, which bind weakly or not at all to heparanoids, fail to elicit arachidonate release, and addition of a heparin binding site to sPLA$_2$-IIC allows it to release arachidonate. Heparin nonbinding sPLA$_2$-X liberates arachidonic acid most likely from the phosphatidylcholine-rich outer plasma membrane in a glypican-independent manner. In rat mastocytoma RBL-2H3 cells that lack glypican, sPLA$_2$-V and -X, which are unique among sPLA$_2$s in being able to hydrolyze phosphatidylcholine-rich membranes, act most likely on the extracellular face of the plasma membrane to markedly augment IgE-dependent immediate production of leukotriene C_4 and platelet-activating factor. sPLA$_2$-IB, -IIA, -IIC, -IID, and -IIE exert minimal effects in RBL-2H3 cells. These results are also supported by studies with sPLA$_2$ mutants and immunocytostaining and reveal that sPLA$_2$-dependent lipid mediator generation occur by distinct (heparanoid-dependent and -independent) mechanisms in HEK293 and RBL-2H3 cells.

Phospholipase A_2 (PLA$_2$),[1] which catalyzes the hydrolysis of membrane glycerophospholipids to produce free fatty acids and lysophospholipids, are a family of intracellular and extracellular enzymes (1–5). Secreted PLA$_2$ (sPLA$_2$) comprises calcium-dependent interfacial enzymes with low molecular mass (typically 14–18 kDa) and multiple disulfides. To date, nine genes coding for structurally related and enzymatically active sPLA$_2$s have been identified in mammals (groups IB, IIA, IIC, IID, IIE, IIF, III, V, and X) (6–10). Understanding the physiological functions of this diverse set of sPLA$_2$s is now a complex and challenging area of research in the eicosanoid field, and the possibility that some of these enzymes are involved in processes unrelated to eicosanoid generation should be considered (11–21).

Group IB sPLA$_2$ (sPLA$_2$-IB), known as pancreatic PLA$_2$, is abundant in pancreatic juice, in which it catalyzes the breakdown of dietary phospholipids, and is also expressed in trace amounts in several tissues including lung and kidney (1, 22). Group IIA sPLA$_2$ (sPLA$_2$-IIA), known as inflammatory PLA$_2$, is expressed in a variety of tissues and hematopoietic cells, and its expression is markedly induced following challenge with proinflammatory stimuli (1, 23–26). This sPLA$_2$ is thought to play a role in inflammation (1), host defense against bacteria (13–15), tumor suppression (16), exocytosis (17, 18), blood coagulation (19), and atherosclerosis (20, 21). Group IIC sPLA$_2$ (sPLA$_2$-IIC) is expressed in rodent testes, but only a pseudogene for this enzyme has been found in the human genome (5, 27). Group V sPLA$_2$ (sPLA$_2$-V) is expressed mainly in rat and human heart (5, 28) and may in part compensate for sPLA$_2$-IIA particularly in the mouse, in which sPLA$_2$-V is inducibly expressed in many tissues by pro-inflammatory agents, whereas sPLA$_2$-IIA expression is largely restricted to mouse intestine (29–31). Group X sPLA$_2$ (sPLA$_2$-X) possesses structural features characteristic of both sPLA$_2$-IB and sPLA$_2$-IIA and is highly expressed in organs associated with the immune response in humans (32).

More recently, several novel mammalian sPLA$_2$s, groups IID, IIE, IIF, and III, have been identified and cloned based on searching nucleic acid data bases for homologs to known mammalian and venom sPLA$_2$s (6–10). Group IID (sPLA$_2$-IID) and IIE (sPLA$_2$-IIE) sPLA$_2$s are structurally most related to sPLA$_2$-IIA, and the genes for these three isozymes as well as those for group IIC, IIF, and V sPLA$_2$s map to the same chromosome locus (4–9). Compared with other group II sPLA$_2$s, group IIF sPLA$_2$ has a relatively long, proline-rich C-terminal extension containing a single cysteine residue and is acidic (8). Group III sPLA$_2$ is a homolog of the group III enzyme originally detected

* This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency, Grant HL36235 from the National Institutes of Health, and by CNRS and the Association pour le Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. Tel,: 81-3-3784-8196; Fax: 81-3-3784-8245; E-mail: kudo@pharm.showa-u.ac.jp.

[1] The abbreviations used are: PLA$_2$, phospholipase A_2; AA, arachidonic acid; Ag, antigen; COX, cyclooxygenase; cPLA$_2$, cytosolic PLA$_2$; FCS, fetal calf serum; IL-1, interleukin-1; LT, leukotriene; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PG, prostaglandin; sPLA$_2$, secreted PLA$_2$; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

in bee venom and possesses long and unique N- and C-terminal extensions (10). Cellular functions of these novel sPLA$_2$s remain to be elucidated. Because the sPLA$_2$ family is diverse and the tissue distribution of each enzyme is unique, these enzymes are likely to have distinct physiological functions.

In an effort to clarify the role of sPLA$_2$s in the regulation of arachidonic acid (AA) release from membrane phospholipids, we have found that sustained expression of sPLA$_2$-IIA or sPLA$_2$-V by forcible gene transfer or by *de novo* induction following cytokine stimulation leads to efficient stimulus-dependent but not spontaneous AA release (24, 33–40). This liberated AA is functionally linked to cyclooxygenase (COX)-mediated prostaglandin (PG) production in several adherent cells (24, 33–40). In such cells, endogenously produced sPLA$_2$-IIA is captured by the heparan sulfate chains of the glycosylphosphatidylinositol-anchored proteoglycan glypican and is transferred to punctate and perinuclear compartments that colocalize with caveolin (39). Such compartmentalization may allow sPLA$_2$-IIA to become in contact with its suitable substrates and to be more efficiently coupled to downstream AA-metabolizing enzymes.

On the other hand, mammalian cells are generally highly resistant to exogenous sPLA$_2$-IIA, with very high concentrations (greater than or equal to 10 μg/ml) usually being required to elicit AA release (41–43). This action has been reported to occur independently of the association of sPLA$_2$-IIA with heparan sulfate proteoglycan (43). Several hematopoietic cells are reportedly more sensitive to exogenous sPLA$_2$-V than sPLA$_2$-IIA (31, 44), and exogenous sPLA$_2$-X is highly active in releasing fatty acids, even from a variety of adherent cells that are refractory to sPLA$_2$-IB and -IIA (45, 46). These diverse features of sPLA$_2$ action may in part reflect their different interfacial binding capacities to charge-neutral phosphatidylcholine (PC) *versus* anionic phospholipid vesicles (43–47). Binding of sPLA$_2$s to PC may be important for the action on the external leaflet of mammalian cells because this membrane surface is rich in charge-neutral PC and sphingomyelin. Indeed, sPLA$_2$-V and -X are able to efficiently hydrolyze PC-rich vesicles *in vitro* (40, 44–46), whereas PC-rich vesicles are a very poor substrate for sPLA$_2$-IIA because of poor binding of this latter enzyme to the interface (43, 47).

sPLA$_2$s display very distinct heparanoid and membrane binding properties, and it is likely that these properties dictate their behavior in various mammalian cells. To better understand the regulatory functions of sPLA$_2$s in lipid mediator biosynthesis, we have extended our gain-of-function studies by transfecting human embryonic kidney 293 (HEK293) cells and rat mastocytoma RBL-2H3 cells with a variety of sPLA$_2$s. Studies using sPLA$_2$ mutants with altered heparanoid and interfacial binding properties provide additional data that help us to formulate models for the mechanisms of action of sPLA$_2$s in these mammalian cells. Moreover, using RBL-2H3 cells, we have demonstrated, for the first time, the functional coupling between specific sPLA$_2$s and the leukotriene (LT) and platelet-activating factor (PAF) biosynthetic pathways.

EXPERIMENTAL PROCEDURES

Materials—HEK293 cells (Human Science Research Resources Bank) and RBL-2H3 cells (Riken Cell Bank) were cultured in RPMI 1640 (Nissui Pharmaceutical Co.) containing 10% fetal calf serum (Bioserum) as described previously (18, 37–40). The cDNAs for mouse sPLA$_2$-IIA and its mutant IIA-KE4 (35), rat sPLA$_2$-V and its mutant V-G30S (37), rat sPLA$_2$-IIC, human sPLA$_2$-X and its mutant X-G30S (40), rat glypican-1 (39), human COX-1 and -2 (38), all of which were subcloned into pcDNA3.1 (Invitrogen), were described previously. The cDNAs for mouse sPLA$_2$-IID (7), human sPLA$_2$-IIA, and its mutants IIA-V3W and R7E/K10E/K16E (43, 47), and human sPLA$_2$-V and its mutant V-W31A (44) were subcloned into pCI-neo (Promega). Mouse sPLA$_2$-IIE cDNA (8) was subcloned into pcDNA3.1(+)/hygro (Invitrogen). Rat sPLA$_2$-IB cDNA was obtained by polymerase chain reaction using rat stomach cRNA as a template with a set of 23-base pair oligonucleotide primers corresponding to 5′- and 3′-nucleotide sequences of the open reading frame and subcloned into pCR3.1 (Invitrogen). C-terminally FLAG-tagged rat sPLA$_2$-V, which was subcloned into pCR3.1, was described previously (29). Mouse cytosolic PLA$_2$ (cPLA$_2$) cDNA was subcloned into pBK-CMV (Stratagene) (37, 52). Site-directed mutagenesis was carried out directly on the mammalian expression plasmids using the QuickChange kit (Stratagene), and all plasmids were submitted to DNA sequencing of the full sPLA$_2$ insert to confirm their sequences.

Rabbit anti-human sPLA$_2$-IIA antibody and the enzyme immunoassay kits for PGE$_2$ and LTC$_4$ were purchased from Cayman Chemicals. The rabbit anti-human COX-1, rabbit anti-human cPLA$_2$, and goat anti-human COX-2 antibodies were purchased from Santa Cruz. Human IL-1β was purchased from Genzyme. LipofectAMINE Plus reagent, Opti-MEM medium and TRIzol reagent were obtained from Life Technologies, Inc. RPMI 1640 medium was purchased from Nissui Pharmaceuticals. Heparin and *Flavobacterium heparinum* heparinase III were purchased from Sigma. Fluorescein isothiocyanate-conjugated goat anti-rabbit and -mouse IgG antibodies were purchased from Zymed Laboratories Inc. Mouse monoclonal anti-FLAG antibody was from Sigma. Mouse IgE anti-trinitrophenyl and trinitrophenyl-conjugated bovine serum albumin were provided by Dr. H. Katz (Harvard Medical School).

Preparation of Recombinant sPLA$_2$s—Recombinant human sPLA$_2$-IIA, mouse sPLA$_2$-IID, and human sPLA$_2$-X were produced in *Escherichia coli* as described (7, 43, 45). Methods for the recombinant expression in *E. coli*, refolding, and purification of mouse sPLA$_2$-IIE will be reported elsewhere.[2] All recombinant sPLA$_2$s were found to be > 95% pure when analyzed by SDS-polyacrylamide gel electrophoresis and to have the predicted mass (within 0.5 atomic mass unit) when analyzed by electrospray mass spectrometry (7, 43, 45). Because the resolution of the instrument in this mass range is 0.5–1 atomic mass unit, mass spectrometry analysis establishes that all sPLA$_2$ have intact disulfides and thus are likely to be properly folded.

Preparation of Antibodies against sPLA$_2$s-IID, -IIE, and -X—Anti-sPLA$_2$ antisera were prepared in rabbits by Cocalico Biologicals Inc. (Reamstown, PA) using an initial injection of 300 μg of sPLA$_2$ in complete Freund's adjuvant followed by a booster injection with 150 μg of sPLA$_2$. Antisera were screened by immunoblotting, and a second boost injection was carried out as needed. Antisera were tested for sPLA$_2$ cross-reactivity using the set of recombinant proteins (human and mouse sPLA$_2$-IB and -IIA, mouse sPLA$_2$-IIC, -IID, -IIE, and -IIF and human sPLA$_2$-X). No cross-reactivity was observed by immunoblotting using 50 ng of each sPLA$_2$ and ECL detection (Amersham Pharmacia Biotech).

Establishment of Transfectants—Establishment of various HEK293 cell transformants was described previously (37–40). Briefly, 1 μg of plasmid was mixed with 2 μl of LipofectAMINE Plus in 100 μl of Opti-MEM medium for 30 min and then added to cells that had attained 40–60% confluence in 12-well plates (Iwaki) containing 0.5 ml of Opti-MEM. After incubation for 6 h, the medium was replaced with 1 ml of fresh culture medium comprising RPMI 1640 containing 10% (v/v) fetal calf serum (FCS). After overnight culture, the medium was replaced again with 1 ml of fresh medium, and culture was continued at 37 °C in an incubator flushed with 5% CO$_2$ in humidified air. The cells were cloned by limiting dilution in 96-well plates in culture medium supplemented with 1 mg/ml geneticin (Invitrogen) or 50 μg/ml hygromycin (Invitrogen). After culture for 3–4 weeks, wells containing a single colony were chosen, and the expression of each protein was assessed by RNA blotting or immunoblotting. The established clones were expanded and used for the experiments as described below.

To establish sPLA$_2$-IID/COX double transformants, HEK293 transformants expressing each COX were subjected to a second transfection with mouse sPLA$_2$-IID cDNA that had been subcloned into pcDNA3.1/Zeo (+) (Invitrogen). Three days after transfection, the cells were used for the experiments or seeded into 96-well plates and cloned by culturing in the presence of 50 μg/ml zeocin (Invitrogen) to establish stable transformants. A similar strategy was employed to produce sPLA$_2$s/glypican-1 double transformants, where cells expressing each sPLA$_2$ were transfected with glypican-1 cDNA in pcDNA3.1/Zeo (+) and selected with zeocin.

RBL-2H3 cells were seeded into 150-mm diameter dishes and cultured for 2~3 days to subconfluency. The cells (10^7 cells) were har-

[2] M. H. Gelb, unpublished observations.

vested, washed twice with Opti-MEM, and suspended in 400 μl of Opti-MEM. The cells were mixed with each cDNA (2~5 μg) and subjected to electroporation (BTX electroporator ECM600, at 200 V pulse amplitude; capacitance, 900 microfarads). After culturing for 2 days, the cells were resuspended in 10 ml of culture medium containing 800 μg/ml geneticin and seeded into 96-well plates. After culture for 2 weeks, single colonies were expanded into 12-well plates. After reaching confluence, the expression of each PLA_2 was assessed by RNA blotting or immunoblotting. As a control, cells transfected with the empty pcDNA3.1 vector were used.

Measurement of sPLA$_2$ Activity—Rates of hydrolysis of phospholipid vesicles by sPLA$_2$s *in vitro* were obtained with the fatty acid binding protein assay as described (45). Reactions contained 30 μM 1-palmitoyl-2-oleoyl-phosphatidylglycerol vesicles (Avanti Polar Lipids Inc.) as 100 nm unilamellar vesicles (prepared by extrusion) in 1.3 ml of Hanks' balanced salt solution with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 9.7 μg of rat liver fatty acid binding protein, and 1 μM 11-dansyl-undecanoic acid (Molecular Probes Inc.) with stirring at 37 °C.

Alternatively, sPLA$_2$ activity was assayed by measuring the amounts of free radiolabeled fatty acids released from the substrate 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine (Amersham Pharmacia Biotech). Each reaction mixture (total volume, 250 μl) consisted of appropriate amounts of the required sample, 100 mM Tris-HCl (pH 7.4), 4 mM $CaCl_2$, and 2 μM substrate. After incubation for 10–30 min at 37 °C, [^{14}C]AA was extracted, and radioactivity was quantified as described previously (35, 48).

Heparin Binding—Affinity of recombinant mouse sPLA$_2$-IID and mouse sPLA$_2$-IIE to heparin-Sepharose was assessed as described previously (35, 37, 40).

RNA Blotting—Approximately equal amounts (~10 μg) of total RNA obtained from transfected cells were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with the respective cDNA probes that had been labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech) by random priming (Takara Biomedicals). All hybridizations were carried out as described previously (35).

SDS-Polyacrylamide Gel Electrophoresis /Immunoblotting—Lysates from 10^5 cells or culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis using 15% (w/v) gels for sPLA$_2$s and 10% gels for COXs under nonreducing and reducing conditions, respectively. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotter (MilliBlot-SDE system; Millipore). The membranes were probed with the respective antibodies (1:2,000 dilutions for sPLA$_2$s and 1:5000 dilutions for COXs) for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000 dilution) (Amersham Pharmacia Biotech) for 2 h, and visualized using the ECL Western blot system (PerkinElmer Life Sciences), as described previously (35).

Activation of HEK293 Cells—HEK293 cells (5×10^4/ml) were seeded into each well of 24- or 48-well plates. To assess AA release (37–40), 0.1 μCi/ml [^{3}H]AA (Amersham Pharmacia Biotech) was added to the cells in each well on day 3, when they had nearly reached confluence, and culturing was continued for another day. After three washes with fresh medium, 250 μl (24-well plate) or 100 μl (48-well plate) of RPMI 1640 with or without 10 μM A23187 (Calbiochem) with 1% FCS or 1 ng/ml IL-1β and/or 10% FCS was added to each well, and the amount of free [^{3}H]AA released into the supernatant during culturing for 0.5 and 4 h, respectively, was measured. The percentage release of AA was calculated using the formula [S/(S + P)] $\times$ 100, where S and P are the radioactivity measured in equal portions of the supernatant and cell pellet, respectively. The supernatants from replicate cells were subjected to the PGE_2 enzyme immunoassay.

Activation of RBL-2H3 Cells—The cells (5×10^4 cells/ml) were seeded into 24-well plates and cultured for 2 days in 1 ml of culture medium. Then the cells were sensitized with IgE anti-trinitrophenyl for 30 min, washed twice, and activated for 10 min at 37 °C with 10 ng/ml trinitrophenyl-conjugated bovine serum albumin as an antigen (Ag) (18). After harvesting the supernatants, the remaining cells were collected and disrupted by two freeze-thawing cycles. Release of LTC_4 was assessed by enzyme immunoassay according to the manufacturer's instruction.

Detection of PAF—RBL-2H3 cells (5×10^6 cells) were preincubated for 10 min with 25 μCi/ml [^{3}H]sodium acetate (PerkinElmer Life Sciences) and then activated for various periods with IgE/Ag in the continued presence of [^{3}H]sodium acetate. After stopping the reaction by adding 0.1% sodium dodecyl sulfate, the lipids contained in the cells and/or supernatants were extracted by the method of Bligh and Dyer (49) and developed on thin layer chromatography plates, as described previously (50). The spot corresponding to PAF was identified by comparison with an authentic PAF standard (Cayman Chemicals), and the silica was scraped from the plate and submitted to scintillation counting.

Confocal Laser Microscopy—Cells grown on collagen-coated cover glasses (Iwaki Glass) were fixed with 3% paraformaldehyde for 30 min in phosphate-buffered saline (PBS). After three washes with PBS, the fixed cells were sequentially treated with 3% bovine serum albumin (for blocking) and 1% saponin (for permeabilization) in PBS for 1 h, with antibodies against each sPLA$_2$ (1:500 dilution) or anti-FLAG antibody (1:200 dilution) for 1 h, and then with fluorescein isothiocyanate goat anti-rabbit or -mouse IgG (1:100 dilution) for 1 h. After six washes with PBS, the cells were mounted on glass coverslips using Perma Fluor (Japan Tanner), and the sPLA$_2$ signal was visualized using a laser scanning confocal microscope (IX70; Olympus), as described previously (18, 39).

Statistical Analysis—Data were analyzed by Student's *t* test. Results are expressed as the means ± S.E., with $p = 0.05$ as the limit of significance.

RESULTS

AA Releasing Function of sPLA$_2$s in HEK293 Cells

Heparanoid Binding—We have recently reported the distinct roles of mouse and human sPLA$_2$-IIA, rat sPLA$_2$-IIC, rat sPLA$_2$-V, and human sPLA$_2$-X in regulating AA metabolism by transfection analyses using HEK293 and Chinese hamster ovary cells as model systems (35, 37–40). To gain more insight into general aspects of the regulatory functions of mammalian sPLA$_2$s, two recently identified sPLA$_2$ enzymes, mouse sPLA$_2$-IID and sPLA$_2$-IIE, were transfected into HEK293 cells. The expression levels of these sPLA$_2$s in stable transfectants, as assessed by RNA blotting, are shown in Fig. 1*A*. When the culture supernatant of cells transfected with mouse sPLA$_2$-IID was applied to a heparin-Sepharose column, the enzyme was recovered from heparin-binding fractions and eluted with a buffer containing 0.7 M NaCl. In contrast, the affinity of mouse sPLA$_2$-IIE for heparin-Sepharose was very weak, a major portion being eluted in the flow-through fraction and only a minor portion (<10%) being eluted from the column with a buffer containing 0.2 M NaCl. The heparin binding affinities of these and other mammalian sPLA$_2$s are compared in Table I. These results are roughly consistent with the calculated pI values for the sPLA$_2$s (IIA > IID ~ V > IIC > IIE > IB > X) (Table I) and thus the degree of positive charge on the sPLA$_2$, which is required for binding to anionic heparanoids. The exception is rat sPLA$_2$-IIC, which has a higher pI value than rat sPLA$_2$-V and yet binds weaker to heparin, showing that the arrangement of basic residues on the surface of the sPLA$_2$ is also important for heparin binding.

More than half of secreted mouse sPLA$_2$-IID was detected in the cell surface-associated fraction that was solubilized with 1 M NaCl as assessed by enzymatic assay and immunoblotting (Fig. 1*B*), indicative of its binding to cell surface heparan sulfate proteoglycan, as reported previously for sPLA$_2$-IIA and -V (Table I and Refs. 35 and 37–40). In contrast, most of the secreted mouse sPLA$_2$-IIE was detected in the supernatant fraction (Fig. 1*B*). Data for these and other sPLA$_2$s are compared in Table I. Treatment of sPLA$_2$-IID-expressing cells with heparin or heparinase solubilized the enzyme into the supernatant (see below), as also observed with mouse sPLA$_2$-IIA (39).

AA Release—When mouse sPLA$_2$-IID-expressing clones were prelabeled with [^{3}H]AA and then stimulated for 30 min with A23187, there was a marked increase in [^{3}H]AA release compared with control cells (Fig. 1*C*). Similarly, when the sPLA$_2$-IID-expressing cells were cultured for 4 h with IL-1 in the presence of FCS, stimulus-dependent but not spontaneous [^{3}H]AA release increased markedly (Fig. 1*D*). In contrast, none

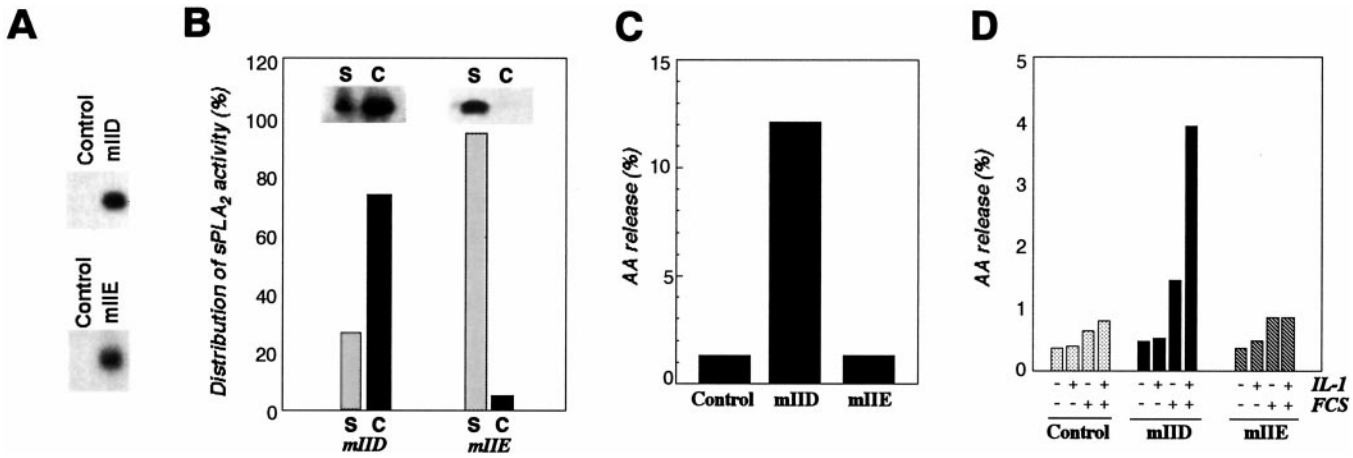

FIG. 1. **Expression of sPLA$_2$-IID and -IIE and their ability to release AA in HEK293 cells.** *A*, RNA blotting of mouse sPLA$_2$-IID (*mIID*) and mouse sPLA$_2$-IIE (*mIIE*) in HEK293 transfectants and parental cells (*Control*). *B*, the distribution of sPLA$_2$s in the supernatants (*S*) and cell surface-associated fractions expressed as a percentage of the total secreted sPLA$_2$ (cell associated plus culture medium; *C*) of transfected HEK293 cells, as determined by enzymatic activity assays. Cells expressing mIID and mIIE, were cultured for 4 days, and then the supernatants containing the secreted enzymes were collected. The cells were then washed for 15 min with 1 M NaCl to solubilize cell surface proteoglycan-associated enzymes (37). *Inset,* immunoblotting of mIID and mIIE in the supernatants (*S*) and cell surface-associated fractions (*C*) of the respective transfectants. *C* and *D*, HEK293 cells transfected with each sPLA$_2$ were prelabeled with [^{3}H]AA and then stimulated for 30 min with 10 μM A23187 (*C*) or for 4 h with or without 1 ng/ml IL-1 and 10% FCS (*D*) to assess [^{3}H]AA release. A representative result of three to six independent experiments is shown.

TABLE I
Properties and eicosanoid generating functions of mammalian sPLA$_2$s in HEK293 cells

sPLA$_2$	Calculated pI	Heparin affinity[a]	Percentage bound to cell surface[b]	AA release[c]	PGE$_2$ formation	Induction of COX-2
IB (rat)	6.96	No binding at 0.15 M	<5	No release	No formation	No
IIA (human)	9.42	0.6–0.8 M (43)	80–90	Immediate and IL-1-dependent delayed release (37)	Immediate via COX-1/2, delayed via COX-2 (37–39)	Yes (39, 40)
IIA (mouse)	9.35	0.7–0.8 M (43)	80–90	Immediate and IL-1-dependent delayed release (37)	Immediate via COX-1/2, delayed via COX-2 (37–39)	Yes (39, 40)
IIC (rat)	8.58	<0.2 M (37)	<5 (37)	No release (37)	No formation (37)	No
IID (mouse)	8.75	0.7 M	70–80	Immediate and IL-1-dependent delayed release	Immediate via COX-1/2, delayed via COX-2	Yes
IIE (mouse)	8.21	<0.2 M	<5	No release	No formation	No
V (human)	8.72	0.4–0.6 M	50–60	Immediate and IL-1-dependent delayed release (37)	Immediate via COX-1/2, delayed via COX-2 (37, 38)	Yes (40)
V (rat)	8.45	0.4–0.6 M (35)	50–60 (37)	Immediate and IL-1-dependent delayed release (37)	Immediate via COX-1/2, delayed via COX-2 (37, 38)	Yes (40)
X (human)	5.10	No binding at 0.15 M (40)	<5 (40)	Immediate and delayed release (IL-1-independent) (40)	No formation unless COX-1 or COX-2 forcibly expressed (40)	No (40)

[a] Concentration of NaCl required for elution of the sPLA$_2$ from a heparin-Sepharose column is given.
[b] Percentage of secreted sPLA$_2$ that is released from HEK293 cells by washing with 1 M NaCl.
[c] HEK293 cells were stimulated with 10 μM A23187 for 30 min (immediate release) or with 1 ng/ml IL-1/10% FCS for 4 h (delayed response).

of the mouse sPLA$_2$-IIE-expressing clones exhibited increased [^{3}H]AA release even after stimulation with A23187 (Fig. 1*C*) or IL-1/FCS (Fig. 1*D*). The AA releasing properties of these and other sPLA$_2$s are compared in Table I. As reported previously (37), catalytic activity is essential for the AA-releasing functions of sPLA$_2$s in HEK293 cells.

PGE$_2$ Biosynthesis—sPLA$_2$-IIA and -V can efficiently couple to stimulus-induced PG biosynthesis via two regulatory steps; enhanced supply of the substrate AA and induction of endogenous COX-2, both of which are required for optimal delayed PG biosynthesis (Refs. 35–40 and Table I). As shown in Fig. 2*A*, mouse sPLA$_2$-IID-transfected, but not control cells, produced a significant amount of PGE$_2$ after stimulation for 4 h with IL-1/FCS. RNA blot analysis showed that sPLA$_2$-IID augmented endogenous COX-2 expression in IL-1-stimulated cells (Fig. 2*B*). Mouse sPLA$_2$-IIE did not elicit PGE$_2$ generation appreciably, most probably because it failed to supply AA and to induce COX-2 (data not shown). The PGE$_2$-generating capacity of these and other sPLA$_2$s in HEK293 cells are compared in Table I.

To investigate functional coupling between mouse sPLA$_2$-IID and COX isozymes further, we carried out cotransfection experiments. The expression levels of sPLA$_2$-IID, COX-1, and COX-2 are shown in Fig. 2*C*. Cells cotransfected with sPLA$_2$-IID and COX-1 produced more PGE$_2$ than those expressing sPLA$_2$-IID or COX-1 alone in response to A23187 (Fig. 2*D*). Similarly, A23187-induced PGE$_2$ production by COX-2-expressing cells was markedly elevated when sPLA$_2$-IID was coexpressed (Fig. 2*D*). When the cells were stimulated with IL-1, PGE$_2$ generation by cells expressing both sPLA$_2$-IID and COX-2 was significantly higher than those expressing sPLA$_2$-IID alone, which linked to endogenously induced COX-2 (Fig.

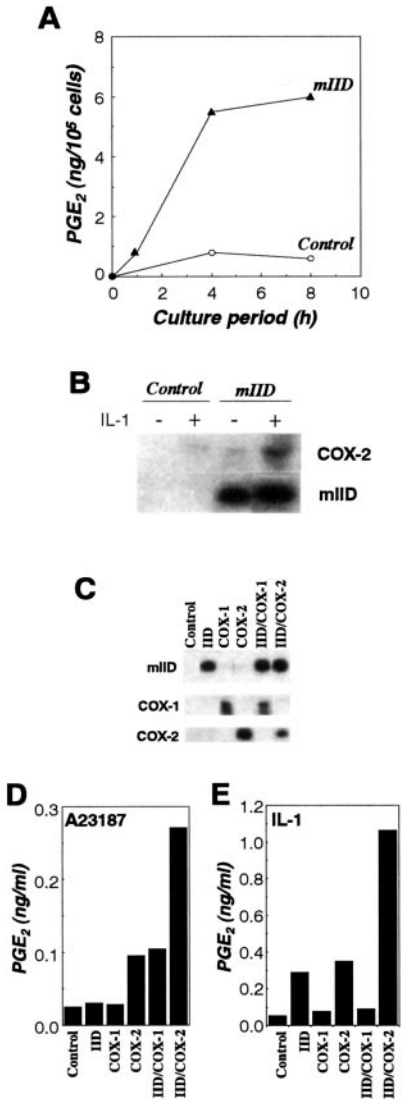

FIG. 2. **PGE$_2$ biosynthesis by mouse sPLA$_2$-IID in HEK293 transfectants.** *A*, cells expressing mouse sPLA$_2$-IID (*mIID*) and control cells were cultured for the indicated periods with 1 ng/ml IL-1 in the presence of 10% FCS in 24-well plates. PGE$_2$ released into the supernatants was quantified. *B*, after 4 h of incubation with (+) or without (−) IL-1/FCS, COX-2 mRNA expression was assessed by RNA blotting. *C*, expression of sPLA$_2$-IID, COX-1 and COX-2 in the transfectants as assessed by RNA blotting. *D* and *E*, PGE$_2$ generation by cells expressing sPLA$_2$-IID and COX-1 or COX-2 alone or in combination. Cells were stimulated for 30 min with A23187 (*D*) or for 4 h with IL-1/FCS (*E*) in 48-well plates, and PGE$_2$ released into the supernatants was quantified. A representative result of three to six independent experiments is shown.

2*B*), or those expressing COX-2 alone (Fig. 2*E*). COX-1 was not utilized in the delayed response even when combined with sPLA$_2$-IID (Fig. 2*E*). In contrast, no appreciable augmentation of PGE$_2$ generation was observed when mouse sPLA$_2$-IIE was cotransfected with each of the two COX isozymes (data not shown), consistent with the fact that sPLA$_2$-IIE expression did not lead to AA release (Fig. 1, *C* and *D*). The functional coupling between these and other sPLA$_2$s with COX isozymes are compared in Table I. Collectively, AA release and COX coupling of sPLA$_2$-IID in the immediate and delayed PGE$_2$-biosynthetic responses are similar to that of the heparin-binding sPLA$_2$-IIA and -V (Refs. 38–40 and Table I).

Gain-of-Function Mutation of sPLA$_2$-IIC—Comparison of the C-terminal domains between sPLA$_2$-IIC and sPLA$_2$-V from various species reveals that the former lacks several basic residues that are conserved in sPLA$_2$-V (Fig. 3*A*). These basic amino acid clusters are important for rat sPLA$_2$-V to bind heparan sulfate proteoglycan on the cell surface and accordingly sPLA$_2$-V-mediated AA release from the transfectants (37). We introduced basic amino acids into the corresponding positions in rat sPLA$_2$-IIC by replacing Leu^{95} and/or Glu^{102} with Arg and Lys, respectively (IIC-L95R and IIC-L95R/E102K), and transfected these mutants into HEK293 cells. Whereas most of the native sPLA$_2$-IIC and IIC-L95R were secreted into the extracellular fluid, 40% of IIC-L95R/E102K was detected in the cell surface-bound fraction (Fig. 3*B*). *In vitro* enzyme activities of native and mutant enzymes did not differ significantly (data not shown). When these transfectants were stimulated with A23187, immediate [^{3}H]AA release by cells expressing IIC-L95R/E102K, but not native enzyme and IIC-L95R, increased markedly (Fig. 3*C*). Furthermore, culturing IIC-L95R/E102K transfectants, but not native enzyme or IIC-L95R transfectants, with IL-1 in combination with FCS resulted in a marked increase in delayed [^{3}H]AA release (Fig. 3*C*). When IIC-L95R/E102K transfectants were cotransfected with either COX-1 or COX-2, A23187-induced immediate PGE$_2$ generation occurred via both COX isozymes, and IL-1-induced delayed PGE$_2$ generation occurred via COX-2 (Fig. 3*D*). There was an increase in PGE$_2$ synthesis in cells expressing IIC-L95R/E102K alone after IL-1 stimulation that was similar to that seen in cells expressing both IIC-L95R/E102K and COX-1 (Fig. 3*D*), suggesting that IIC-L95R/E102K expression induces endogenous COX-2 expression. Thus, IIC-L95R/E102K, which acquired the ability to associate with the cell surface, behaves like sPLA$_2$-IIA, -IID, and -V in regulating PGE$_2$ biosynthesis in HEK293 cells.

Interaction of sPLA$_2$s with Glypican—Functional similarities among the three heparin-binding group II subfamily of sPLA$_2$s (IIA, IID and V) in HEK293 cells suggest that they utilize a common regulatory machinery for AA metabolism. Because the function of sPLA$_2$-IIA depends on its interaction with the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan glypican in these cells (39), we examined whether sPLA$_2$-IID and -V also utilize the glypican-dependent pathway. For this purpose, we transfected glypican-1 cDNA into HEK293 cells expressing mouse sPLA$_2$-IID (Fig. 4) or rat sPLA$_2$-V (Fig. 5*A*). Because the augmentative effect of glypican on sPLA$_2$-IIA function is particularly evident when sPLA$_2$-IIA expression is suboptimal (39), we chose clones expressing low levels of sPLA$_2$-IID (Fig. 4) and -V (Fig. 5*A*) in this experiment.

The expression of sPLA$_2$-IID and glypican-1 in HEK293 cells transfected with their cDNAs, alone or in combination, is shown in Fig. 4*A*. The amount of sPLA$_2$-IID bound on the cell surface increased about 2-fold following glypican-1 coexpression (data not shown). IL-1-stimulated delayed AA release by cells coexpressing sPLA$_2$-IID and glypican was significantly higher than that by cells expressing sPLA$_2$-IID alone (Fig. 4*B*). A similar increase in A23187-induced immediate AA release was also observed following introduction of glypican into sPLA$_2$-IID-expressing cells (data not shown). PGE$_2$ produced by sPLA$_2$-IID/glypican cotransfectants 4 h after IL-1 stimulation reached ~15-fold higher levels than that produced by cells expressing sPLA$_2$-IID alone (Fig. 4*C*). IL-1-induced COX-2 expression, which was elevated modestly in the sPLA$_2$-IID-transfected cells relative to control cells, was further increased in cells coexpressing sPLA$_2$-IID and glypican (Fig. 4*D*), whereas no significant augmentation of COX-2 induction occurred when sPLA$_2$-IID/glypican cotransfectants were cultured in the absence of IL-1 (data not shown). These results imply that enhanced AA release (Fig. 4*B*) and COX-2 induction (Fig. 4*D*) converge on synergistic augmentation of PGE$_2$ generation following IL-1 stimulation (Fig. 4*C*). In further support of the functional inter-

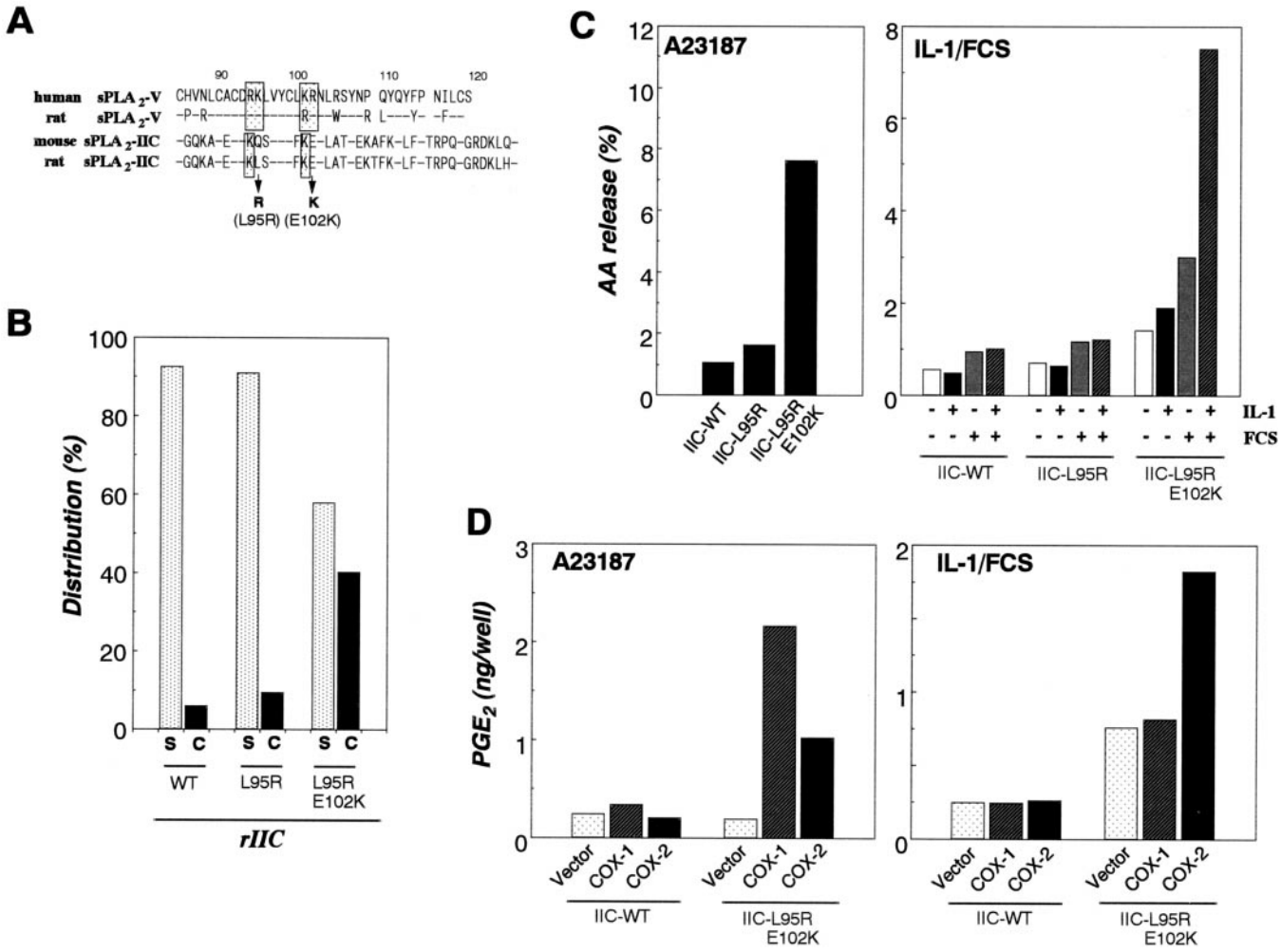

FIG. 3. **Site-directed mutagenesis of sPLA$_2$-IIC.** *A*, alignment of the C-terminal regions of sPLA$_2$-V and -IIC. The conserved cationic amino acids are surrounded by *boxes*. Residues Lsu^{95} and Glu^{102} in rat sPLA$_2$-IIC were mutated to Arg and Lys, respectively. The amino acid numbers are based on the alignment with the sPLA$_2$-IB sequence. *B*, the percentage distribution of sPLA$_2$-IIC and its mutants in the supernatants (*S*) and cell surface-associated fractions (*C*), as assessed by enzymatic activity assay. *WT*, wild type. *C*, cells prelabeled with [^{3}H]AA were stimulated for 30 min with A23187 or for 4 h with or without IL-1/FCS, and AA release was quantified. *D*, cells expressing native or mutant sPLA$_2$-IIC were transiently transfected with either COX-1 or COX-2. Three days after transfection, cells were stimulated for 30 min with A23187 or for 4 h with IL-1/FCS, and PGE$_2$ generation was quantified. Expression of COX-1 and COX-2 was verified by immunoblotting (not shown). A representative result of three independent experiments is shown.

action between sPLA$_2$-IID and glypican, we found that treating the cells with heparin or heparinase markedly reduced IL-1-stimulated PGE$_2$ generation by both sPLA$_2$-IID single and sPLA$_2$-IID/glypican double transfectants (Fig. 4*E*), accompanied by solubilization of sPLA$_2$-IID into the extracellular culture medium (Fig. 4*F*).

As shown in Fig. 5*A*, IL-1-stimulated AA release by cells expressing suboptimal level of rat sPLA$_2$-V was also enhanced markedly by glypican coexpression. This result shows that sPLA$_2$-V, a heparin-binding sPLA$_2$, also utilizes the glypican-dependent pathway in HEK293 cells. In contrast, FCS-dependent AA release by human sPLA$_2$-X, a heparin-nonbinding isozyme, was not influenced appreciably by glypican coexpression (Fig. 5*B*). Thus, the augmentation by glypican is confined to the heparin-binding group II subfamily of sPLA$_2$s and is not a reflection of a nonspecific action on cells.

Immunocytostaining—We have previously shown that sPLA$_2$-IIA overexpressed in HEK293 cells and cytokine-induced endogenous sPLA$_2$-IIA in rat fibroblastic 3Y1 and hepatic BRL-3A cells resides in punctate and perinuclear compartments that colocalize with caveolin (39). Immunocytostaining of mouse sPLA$_2$-IID-expressing 293 cells with its specific antibody revealed positive signals in punctate compartments throughout the cell and in the perinuclear area (Fig. 6, *top panels*). These signals were largely abrogated when cells were incubated with heparin (Fig. 6, *middle panels*). Because heparin is cell impermeable, this result shows that the intracellular punctate domain enzyme is in exchange with secreted enzyme and is not the result of intracellular aggregation of overexpressed sPLA$_2$-IID. These results are indistinguishable from the subcellular localization of sPLA$_2$-IIA (39). In contrast, punctate signals were virtually undetectable when human sPLA$_2$-X-transfected cells were immunostained with its specific antibody (Fig. 6, *bottom panel*). These results are consistent with the idea that sPLA$_2$-IID, as is the case for sPLA$_2$-IIA (39), is localized in caveolae-derived compartments through binding to glycosylphosphatidylinositol-anchored glypican, whereas heparin-nonbinding sPLA$_2$-X is mainly, if not exclusively, released into the extracellular medium. Positive signals near the perinuclear area in sPLA$_2$-X-expressing cells appear to correspond to Golgi, reflecting the sPLA$_2$-X secretion process.

Studies Using Interfacial Binding Site Mutants of sPLA$_2$s—To explore whether the glypican-dependent group II subfamily of sPLA$_2$s (IIA, IID, and V) acts on the PC-rich outer plasma membrane or on another compartmentalized membrane that is assumed to be rich in anionic lipids, we transfected HEK293 cells with human sPLA$_2$-IIA and V mutants that have altered inter-

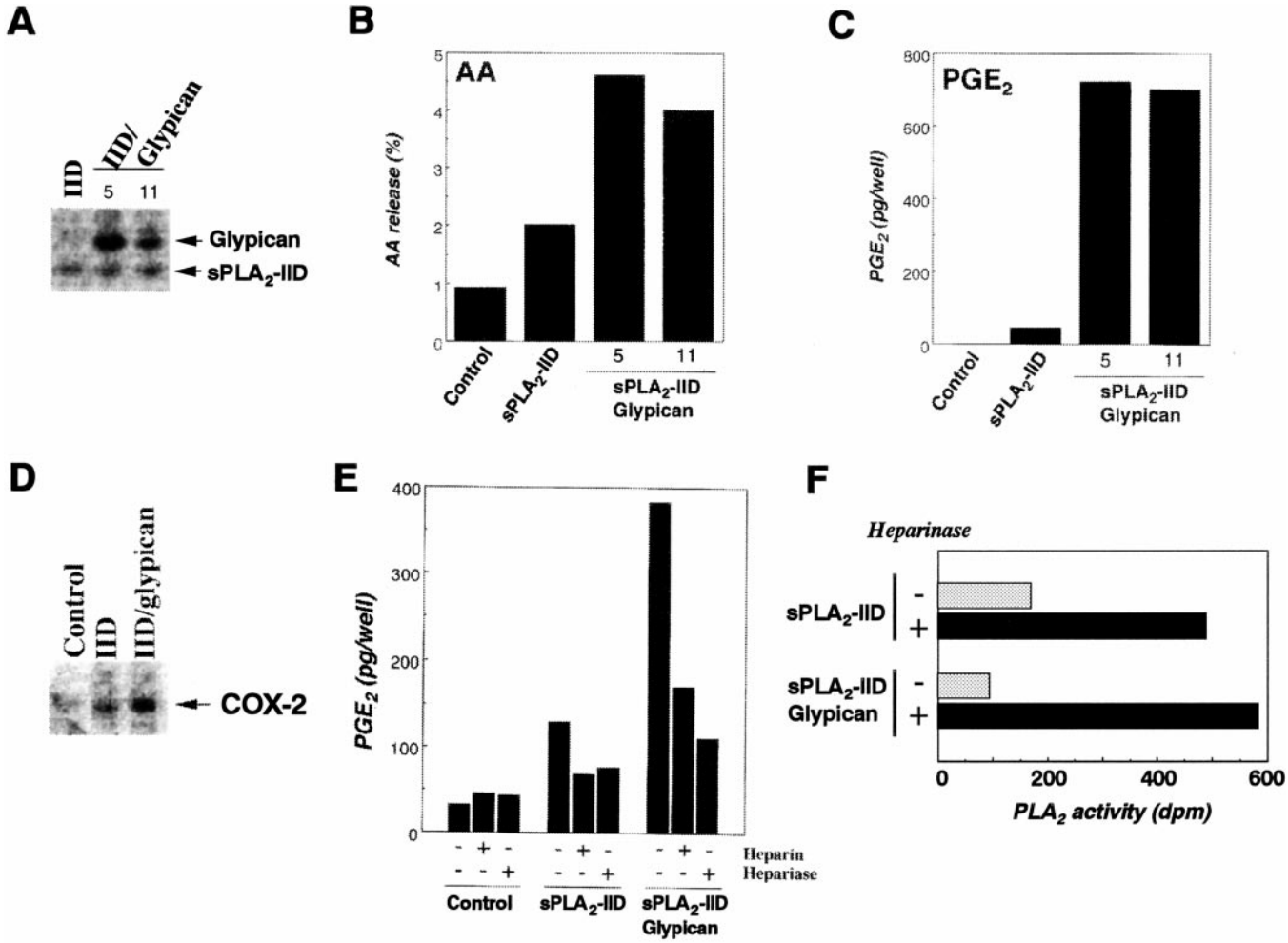

FIG. 4. **Functional interaction of sPLA$_2$-IID with glypican in HEK293 cells.** *A*, expression of mouse sPLA$_2$-IID and glypican in HEK293 cells as assessed by RNA blotting. sPLA$_2$-IID single transfectants and two independent sPLA$_2$-IID/glypican transfectants (clones 5 and 11) are shown. *B–D*, cells were stimulated for 4 h with IL-1/FCS. AA (*B*) and PGE$_2$ (*C*) released into the supernatants were measured. The remaining cells were harvested and subjected to RNA blotting to examine the expression of COX-2 mRNA. *E* and *F*, cells preincubated for 2 days with 1 mg/ml heparin or 0.5 unit/ml heparinase were stimulated for an additional 4 h with IL-1/FCS, and PGE$_2$ activity (*E*) and PLA$_2$ activity (*F*) in the supernatants were quantified. A representative result of three to five independent experiments is shown.

facial binding to zwitterionic PC vesicles *in vitro* (43, 44, 47). When assayed *in vitro* using PC as a substrate, IIA-V3W, in which Val^3 is replaced by Trp, is about 300-fold more active than wild type enzyme (IIA-WT), most likely because the mutant binds more tightly than wild type to PC vesicles (43, 47). Likewise, V-W31A, in which Trp^{31} is replaced by Ala, is about 200-fold less active than V-WT (44). Enzymatic activities of IIA-WT and IIA-V3W toward anionic phospholipids are virtually identical because both bind tightly to anionic vesicles, and the same is true for V-WT and V-W31A (43, 44, 47).

The expression levels of the native and mutant sPLA$_2$s were compared by RNA blotting (Fig. 7*A*), immunoblotting (Fig. 7*B*), and enzyme activity toward anionic vesicles (Fig. 7*C*). These analyses showed that IIA-WT and IIA-V3W were expressed at a similar level (Fig. 7, *A–C*), as were V-WT and V-W31A (Fig. 7, *A* and *C*). These transfectants were prelabeled with [^{3}H]AA and stimulated with A23187 for 30 min (Fig. 7*D*) or with IL-1/FCS for 4 h (Fig. 7*E*). Release of [^{3}H]AA by cells expressing the mutant enzymes did not differ significantly from that released by the cells expressing the respective wild type enzymes. Enzyme assay revealed that more than 90% of IIA-WT and IIA-V3W, and nearly 60% of V-WT and V-W31A were recovered from the cell surface-associated fraction, indicating that these mutations did not significantly alter binding to glypican. Thus, these studies support the idea that the glypican-dependent sPLA$_2$s do not act on the PC-rich extracellular face of the plasma membrane but in a compartment enriched in anionic phospholipids.

We also established transfectants that express another human sPLA$_2$-IIA mutant, IIA-R7E/K10E/K16E, which displays weak heparin and heparan sulfate affinity but unaltered enzymatic activity on anionic vesicles (43). More than 90% of the enzyme activity was released into the supernatant (Fig. 7*C*), indicative of its impaired heparan sulfate binding. IL-1/FCS-stimulated [^{3}H]AA release increased minimally in IIA-R7E/K10E/K16E transfectants compared with IIA-WT expressing cells (Fig. 7*E*), despite the fact that the expression level of this mutant was almost equal to that of IIA-WT (Fig. 7, *A–C*). This result reinforces the importance of heparanoid-binding for sPLA$_2$-IIA function and shows that in addition to the C-terminal lysine cluster (35), several basic residues in the N-terminal helix of sPLA$_2$-IIA can also contribute significantly to functional interaction of sPLA$_2$-IIA with heparan sulfate (43).

AA Releasing Function of sPLA$_2$s in Mast Cells

Granule Accumulation and Secretion—Our previous studies established that heparin-binding (IIA, IID, and V) and non-binding (IIC and IIE) sPLA$_2$s are expressed endogenously in mouse bone marrow-derived mast cells (18). To explore the AA releasing function of these sPLA$_2$s in mast cells and to examine

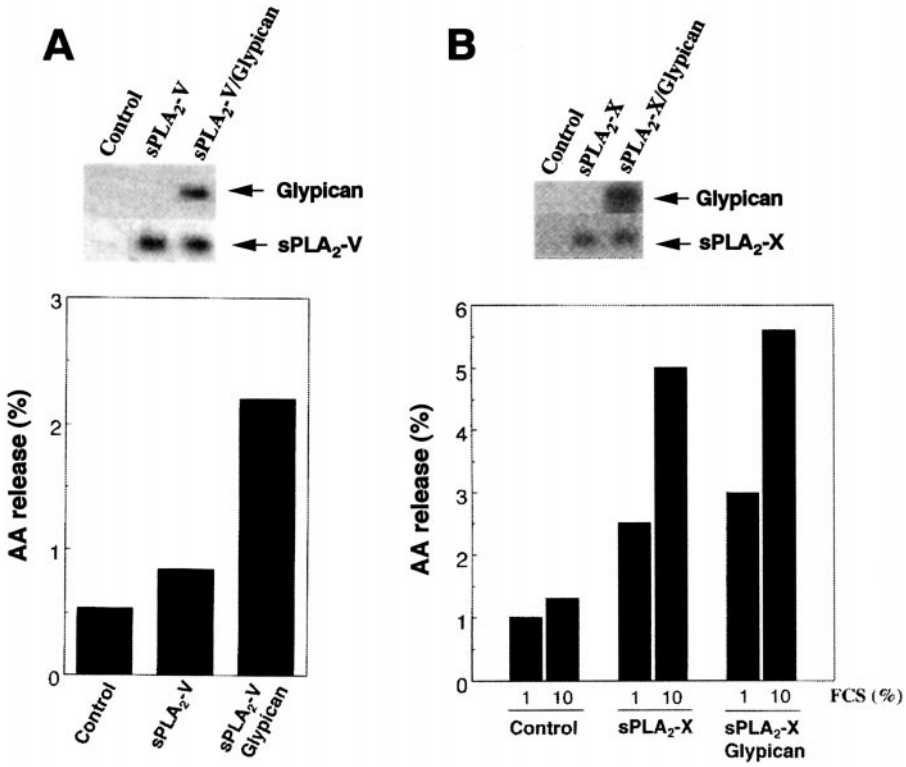

FIG. 5. **Effects of glypican overexpression on the AA-releasing function of sPLA$_2$-V and -X in HEK293 cells.** Glypican was coexpressed with rat sPLA$_2$-V (*A*) and human sPLA$_2$-X (*B*), and AA release from the transfectants in response to IL-1/FCS (*A*) or FCS alone (*B*) was measured. Expression levels of each sPLA$_2$ and glypican in the transfectants, as assessed by RNA blotting, are shown in the *top panel*. A representative result of three independent experiments is shown. Previous studies showed that IL-1 addition does not enhance AA release in HEK293 cells expressing sPLA$_2$-X (40).

possible correlations of *in vitro* properties of sPLA$_2$s with functional properties in mast cells, we overexpressed different mammalian sPLA$_2$s in rat mastocytoma RBL-2H3 cells (the expression of each sPLA$_2$ in the established transfectants is shown in Fig. 8, *insets*). These sPLA$_2$s were rapidly released (within 5 min) after cross-linking of the high affinity IgE receptor by multivalent Ag. The release of sPLA$_2$-IB, -IIA, -IIC, -IID, -V, and -X into the supernatants after IgE/Ag activation reached 50, 5, 45, 15, 23, and 55% (relative to total sPLA$_2$ content in cells), respectively (Table II), whereas spontaneous release of all of these enzymes was minimal, as assessed by enzymatic assay. Lower percentage release of sPLA$_2$-IIA, -IID, and -V *versus* a higher percentage release of -IB, -IIC, and -X appears to reflect the association of the former enzymes with heparanoids or other anionic components on the surface of cells; an idea supported by the observation that IgE/Ag-induced release of the heparin weak binding mouse sPLA$_2$-IIA mutant, KE4 (35), reached a level comparable with that of sPLA$_2$-X (18). Consistent with the idea that caveolae are poorly developed in cells of hematopoietic origin (54–56), no caveolae-like structures were observed by immunocytochemical studies (see below), and the expression of caveolin-2 and glypican-1 was undetectable in RBL-2H3 cells by immunoblotting (data not shown). Therefore, sPLA$_2$-IIA, -IID, and -V may bind to heparanoid proteoglycans other than glypican or to nonheparanoid anionic components on the extracellular surface of activated RBL-2H3 cells.

LTC$_4$ Biosynthesis—Fig. 8 illustrates the functional coupling between sPLA$_2$s and endogenous 5-lipoxygenase for IgE/Ag-induced LTC$_4$ biosynthesis in RBL-2H3 transfectants. LTC$_4$ generation by the transfectants expressing rat sPLA$_2$-IB (Fig. 8*A*), mouse (Fig. 8*B*), rat, and human sPLA$_2$-IIA (data not shown), rat sPLA$_2$-IIC (Fig. 8*C*), mouse sPLA$_2$-IID (Fig. 8*D*), and mouse sPLA$_2$-IIE (Fig. 8*E*) increased only minimally compared with that produced by mock transfected cells. In contrast, cells expressing rat sPLA$_2$-V (Fig. 8*F*) and human sPLA$_2$-X (Fig. 8*G*) produced significant amounts of LTC$_4$, reaching levels comparable with that produced by cells transfected with cPLA$_2$ (Fig. 8*H*), which has been shown by gene disruption to be crucial for LTC$_4$ generation in mast cells (57). The expression of endogenous 5-lipoxygenase, 5-lipoxygenase-activating protein and LTC$_4$ synthase, as assessed by RNA blotting and immunoblotting, did not differ significantly among the transfectants used (data not shown), indicating that the LTC$_4$-biosynthetic effect of sPLA$_2$-V and -X expression was not due to an alteration in the expression of downstream enzymes in the 5-lipoxygenase pathway. Catalytic site mutants V-G30S (Fig. 9*A*) and X-G30S (Fig. 9*B*) with very low enzymatic activity, in which Gly^{30} in the Ca^{2+} binding loop of rat sPLA$_2$-V (37) and human sPLA$_2$-X (40), respectively, is replaced by Ser, failed to augment LTC$_4$ generation, indicating that a functional catalytic site is essential.

Studies Using Interfacial Mutants of sPLA$_2$s—Because sPLA$_2$-V and -X show high interfacial binding to PC vesicles *in vitro* (44, 45), whereas the other sPLA$_2$s do not,[2] we reasoned that their potent LTC$_4$-biosynthetic activity might be a reflection of their action on the PC-rich outer plasma membrane after exocytosis. To explore this hypothesis, we examined the effect of the human sPLA$_2$-V mutant V-W31A with impaired PC vesicle binding (44) on LTC$_4$ generation. As shown in Fig. 9*C*, cells transfected with V-W31A, the expression of which was even higher than that of native sPLA$_2$-V, produced minimal LTC$_4$. Conversely, transfection of the human sPLA$_2$-IIA mutant IIA-V3W, which has increased affinity for PC (43, 47), led to a 5-fold increase in IgE/Ag-induced LTC$_4$ biosynthesis relative to that produced in wild type sPLA$_2$-IIA-expressing cells (0.10 and 0.51 ng/10^6 cells in IIA-WT and IIA-V3W-transfected cells, respectively). Thus, unlike PGE$_2$ generation in HEK293 cells shown above, LTC$_4$ generation by sPLA$_2$s in mast cells correlates with their ability to bind PC vesicles and does not correlate with their heparin-binding affinity.

PAF Biosynthesis—IgE/Ag-induced activation of mast cells leads to production of another lipid mediator PAF via the *sn*-2 ester hydrolysis of 1-*O*-alkyl-PC by PLA$_2$ and subsequent acetylation by PAF acetyltransferase. When RBL-2H3 cells transfected with various sPLA$_2$s were stimulated with IgE/Ag,

the production of PAF in cells overexpressing the PC-hydrolyzing isozymes rat sPLA$_2$-V and human sPLA$_2$-X increased, reaching a level comparable with that in cells overexpressing cPLA$_2$ (Fig. 10). cPLA$_2$ has been shown to be involved in PAF biosynthesis from studies using cPLA$_2$-null mice (51). In contrast, expression of mouse sPLA$_2$-IIA, -IID (Fig. 10), and -IIE, rat sPLA$_2$-IB and -IIC, and the human sPLA$_2$-V mutant V-W31A (data not shown) did not lead to augmentation of PAF generation. These results collectively suggest that the hydrolysis of PC by sPLA$_2$-V or -X leads to release of AA and lyso-PAF, which are supplied to 5-lipoxygenase as a substrate for LTC$_4$ biosynthesis and to PAF acetyltransferase for PAF biosynthesis, respectively. The ability of sPLA$_2$s to promote immediate generation of PGD$_2$, LTC$_4$, and PAF and to augment degranulation in RBL-2H3 cells is summarized in Table II.

Immunocytostaining—We have recently shown by confocal and electron microscopic analyses that sPLA$_2$-IIA is stored in secretory granules of unstimulated RBL-2H3 transfectants and moves in close proximity to the plasma membrane after IgE/Ag activation, the area corresponding to opening perigranular membranes where fusion between the plasma and granule membranes is occurring (18). This particular localization is dependent on the binding of sPLA$_2$-IIA to an unidentified anionic cell component, possibly a heparan sulfate proteoglycan other than glypican. This compartmentalization of sPLA$_2$-IIA may lead to spatially segregated lysophospholipid production, which may enhance membrane fusion leading to degranulation (18).

To further elucidate the sites of action of sPLA$_2$-V and -X in mast cells, RBL-2H3 cells were transiently transfected with C-terminally FLAG-tagged rat sPLA$_2$-V and native human sPLA$_2$-X, and transfectants were examined by confocal laser immunofluorescent microscopy using anti-FLAG antibody and anti-sPLA$_2$-X antiserum, respectively. Like sPLA$_2$-IIA (18), sPLA$_2$-V also resides in granular components in the cytoplasm of cells before IgE/Ag activation (Fig. 11), confirming its localization in secretory granules. After cell activation, sPLA$_2$-V gave a signal somewhat different from that of sPLA$_2$-IIA (18). Only the outline of the sPLA$_2$-V expressing cells was intensely stained by the anti-FLAG antibody (Fig. 11). This result implies that sPLA$_2$-V is exocytosed and then bound over the entire plasma membrane surface. Possibly, the weaker affinity of sPLA$_2$-V for heparan sulfate compared with that of sPLA$_2$-IIA may allow sPLA$_2$-V to disperse from the perigranular membrane, to which sPLA$_2$-IIA binds (18), onto the plasma membrane surface, where it may associate with PC and with some other unknown proteoglycan species or anionic components.

Immunofluorescence studies with sPLA$_2$-X-transfected cells revealed that this enzyme is also stored in granular components prior to IgE/Ag activation (Fig. 11). After cell activation, weak staining of the plasma membrane was observed. This staining pattern is in line with the observation that a large portion of sPLA$_2$-X is secreted extracellularly (see above) and with the idea that its association with the cell surface depends on its interfacial binding to the PC-rich membrane. Collectively, these immunocytochemical studies further support the idea that in activated mast cells, exocytosed sPLA$_2$-V and -X interact with the PC-rich outer leaflet of the plasma membrane to liberate AA and lysoPAF, which are supplied to downstream enzymes for lipid mediator biosynthesis.

DISCUSSION

In this study, we have analyzed the AA-releasing function and attendant lipid mediator-producing capacity of a collection of mammalian sPLA$_2$s in two transfected cell lines where sPLA$_2$s display different profiles of secretion and localization. In HEK293 cells, sPLA$_2$s enter the constitutive secretory process. This pathway appears to be reminiscent of fibroblasts (24), hepatocytes (34), mesangial cells (23, 25), smooth muscle cells (58, 59), and endothelial cells (33), in which expression of sPLA$_2$-IIA for example, is up-regulated by proinflammatory stimuli and is maintained over a long culture period. In this system, heparin-binding sPLA$_2$s (IIA, IID, and V) bind to glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan glypican and possibly other cell surface components and accumulate in caveolin-rich and perinuclear compartments (Ref. 39 and Fig. 6), where they augment stimulus-induced AA release and PGE$_2$ generation. This heparan sulfate proteoglycan-dependent action occurs independently of their interfacial affinity for PC vesicles (Fig. 7). In contrast, in mastocytoma RBL-2H3 cells, sPLA$_2$-IIA (18), -V, and -X (Fig. 11) are stored in secretory granules and are released immediately after cell activation through the degranulation pathway of secretion. The fact that sPLA$_2$-IB, -IIC, and -IID are also rapidly released from IgE/Ag-stimulated RBL-2H3 cells suggests that these enzymes also reside in secretory granules. This route often takes place in hematopoietic cells such as mast cells (18, 60), platelets (61), and neutrophils (62). In RBL-2H3 cells, only sPLA$_2$-V and -X are capable of augmenting LTC$_4$ and PAF generation for reasons discussed below. In some cells, both degranulation and constitutive secretion may occur. The schematic models for the two pathways are illustrated in Fig. 12.

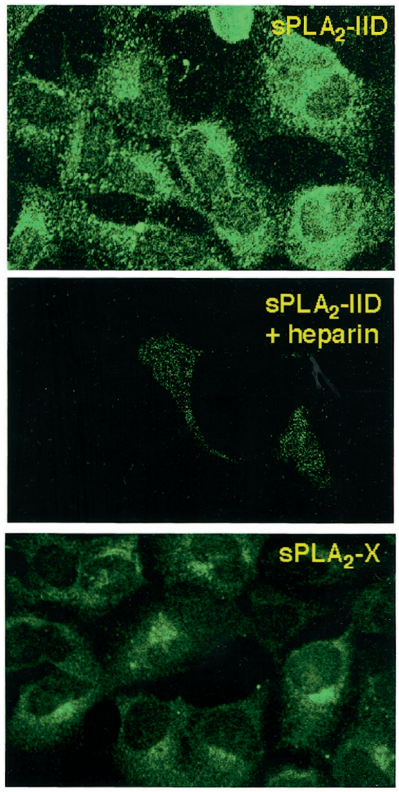

FIG. 6. **Immunocytostaining of sPLA$_2$-IID and -X in HEK293 transfectants.** Cells expressing mouse sPLA$_2$-IID (*top and middle panels*) and human sPLA$_2$-X (*bottom panel*) were fixed, permeabilized, and subjected to immunostaining using specific antibodies as detailed under "Experimental Procedures." In the *middle panel*, sPLA$_2$-IID-expressing cells are cultured for 4 h in the presence of 1 mg/ml heparin.

Heparanoid-dependent Action—The following consistent picture is emerging for the action of heparin-binding sPLA$_2$s (IIA, IID, and V) in promoting eicosanoid generation in cells that utilize the glypican shuttling mechanism. Binding of heparin-binding sPLA$_2$s to the heparan sulfate chains of glypican allows accumulation of the enzyme on the cell surface and also

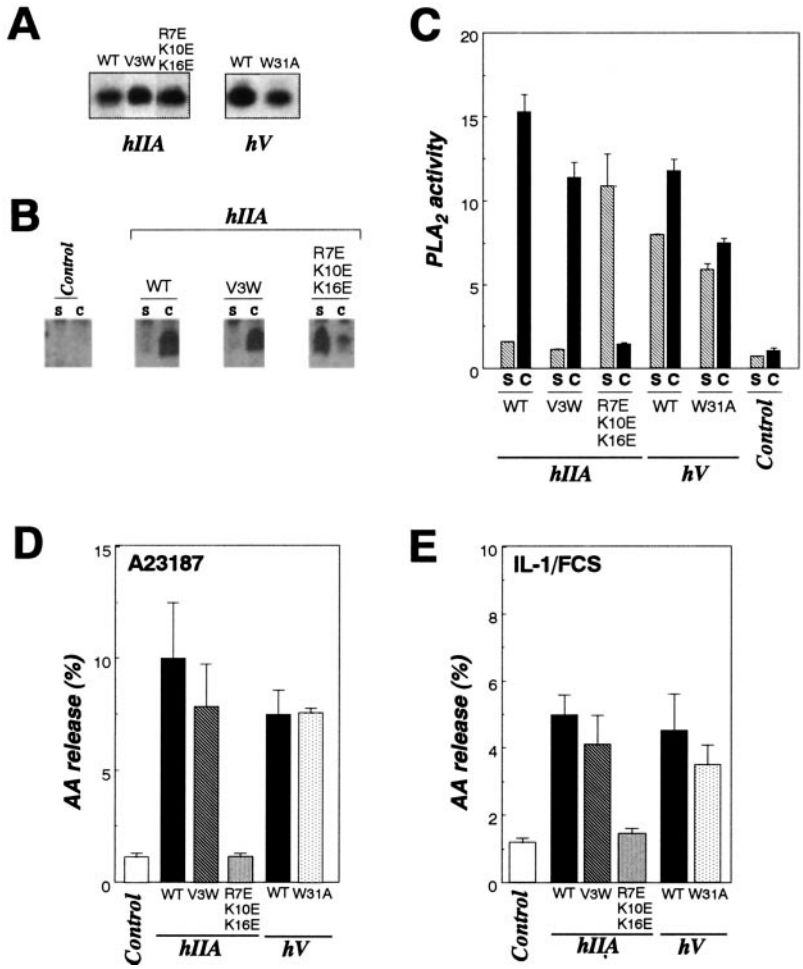

FIG. 7. **Effects of mutation of the interfacial binding surfaces of sPLA$_2$-IIA and -V on AA release in HEK293 cells.** *A*, expression of wild type (*WT*) and mutant human sPLA$_2$-IIA and -V in HEK293 cells, as assessed by RNA blotting. *B* and *C*, distribution of sPLA$_2$-IIA-WT and three mutants in the supernatants (*S*) and cell surface-associated fractions (*C*), as assessed by immunoblotting using anti-sPLA$_2$-IIA antibody (*B*) and enzymatic activity toward phosphatidylglycerol vesicles measured by the fatty acid binding protein assay (*C*). *D* and *E*, cells expressing wild type or mutant sPLA$_2$-IIA and -V were stimulated for 30 min with A23187 (*D*) or for 4 h with IL-1/FCS (*E*), and AA release was quantified. Values are the means ± s.e. of three to five experiments

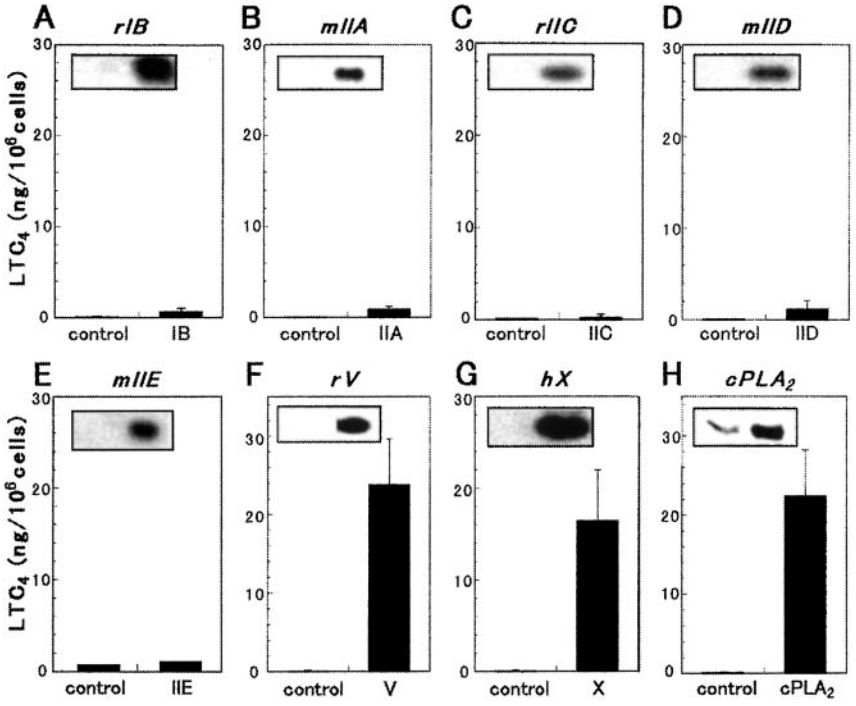

FIG. 8. **Effects of various sPLA$_2$s on IgE/Ag-mediated LTC$_4$ generation in RBL-2H3 transfectants.** RBL-2H3 cells were stably transfected with rat sPLA$_2$-IB (*rIB*; *A*), mouse sPLA$_2$-IIA (*mIIA*; *B*), rat sPLA$_2$-IIC (*rIIC*; *C*), mouse sPLA$_2$-IID (*mIID*; *D*), mouse sPLA$_2$-IIE (*mIIE*; *E*), rat sPLA$_2$-V (*rV*; *F*), human sPLA$_2$-X (*hX*; *G*), and mouse cPLA$_2$ (*H*). The cells were sensitized with IgE and stimulated for 10 min with Ag as described under "Experimental Procedures." LTC$_4$ released into the supernatants was quantified. The expression levels of PLA$_2$s, assessed by RNA blotting (for sPLA$_2$s) and immunoblotting (for cPLA$_2$), are shown in the *insets*. Values are the means ± S.E. of three to seven independent experiments.

promotes enzyme internalization into punctate domains that are rich in caveolin.

On the other hand, sPLA$_2$-IB, -IIC, and -IIE, which have low affinity for heparin, are found mainly in the culture medium rather than bound to the cell surface, and they failed to elicit AA release under the conditions employed here. Clusters of basic amino acids near the C and N termini of sPLA$_2$-IIA and -V form the binding sites for negatively charged heparin or heparan sulfate (35, 37, 43, 63). sPLA$_2$-IIA and -V mutants in which these basic amino acids are mutated show reduced hep-

TABLE II
Properties and functions of mammalian sPLA$_2$s in RBL-2H3 cells

sPLA$_2$	Percentage secreted upon IgE/Ag stimulation	Degranulation (18)	PGD$_2$ generation (18)[a]	LTC$_4$ generation[a]	PAF formation[a]
IB (rat)	50	Weak enhancement	Weak	No	No
IIA (human)	ND[b]	Enhancement	No	No	ND
IIA (mouse)	5	Enhancement	No	No	No
IIC (rat)	45	No enhancement	No	No	No
IID (mouse)	15	Enhancement	Weak	No	No
IIE (mouse)	ND	No enhancement	No	No	No
V (human)	ND	Enhancement	Yes	Yes	ND
V (rat)	23	Enhancement	Yes	Yes	Yes
X (human)	55	Weak enhancement	Yes	Yes	Yes

[a] RBL-2H3 cells were stimulated with IgE and antigen (Ag) for 10 min (immediate release).
[b] ND, not determined.

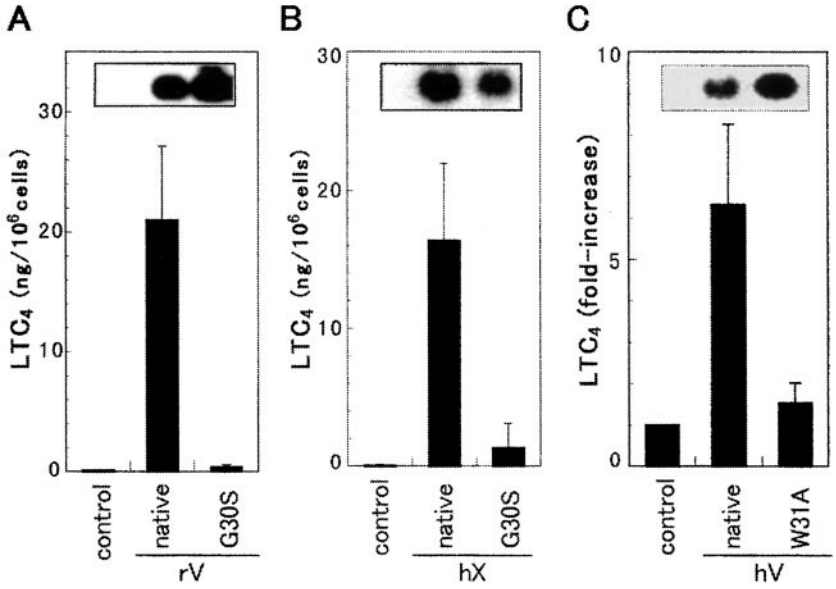

FIG. 9. **Effects of sPLA$_2$-V and -X mutants on LTC$_4$ generation in RBL-2H3 cells.** Cells were transfected with wild type or mutant sPLA$_2$s, and human sPLA$_2$-X (*B*), and IgE/Ag-dependent LTC$_4$ generation was quantified. Values are the means ± S.E. of three to six independent experiments.

arin affinity and lose their ability to elicit AA release (35, 37, 43). sPLA$_2$-IID contains this basic amino acid cluster in the C-terminal domain (7), whereas fewer basic residues are found in the corresponding portions of sPLA$_2$-IIC (27) and -IIE (8). Our ability to functionally manipulate the behavior of sPLA$_2$s by protein engineering, *i.e.* loss-of-function by removal of the glypican binding (sPLA$_2$-IIA and -V) and gain-of-function by addition of a heparin binding site (sPLA$_2$-IIC), provides very strong circumstantial evidence for the functional requirement of glypican shuttling in IL-1/FCS-dependent AA liberation and PGE$_2$ production in the HEK293 cell model.

We cannot rule out the possibility that the heparan sulfate mechanism only occurs as a result of sPLA$_2$ overexpression in transfected cells. However, the physiological significance of heparan sulfate-dependent action of sPLA$_2$-IIA is supported by the observations that with other cells such as human umbilical vein endothelial cells (33), rat hepatocytic BRL-3A cells (34), and rat fibroblastic 3Y1 cells (24), solubilization of membrane surface-associated endogenous sPLA$_2$-IIA by exogenous heparin or heparinase greatly reduced cytokine-stimulated prolonged PG biosynthesis. Furthermore, in BRL-3A and 3Y1 cells, cytokine-induced endogenous sPLA$_2$-IIA is colocalized with caveolin (39).

As a result of glypican shuttling in these cells, endogenously expressed sPLA$_2$-IIA is delivered into a caveolae-like compartment and internalized through potocytosis to reach the perinuclear area (39), where the downstream PG-biosynthetic enzymes (COX and PGE$_2$ synthase) are located (64, 65). This sPLA$_2$-IIA sorting appears to be crucial for its proper function. Exogenously added sPLA$_2$-IIA, which is poorly active on mammalian cells (1, 41–43), does not access this shuttling process for reasons that are not yet clear. It has been reported that in certain cells, exogenously added sPLA$_2$-IIA deposits poorly on cell surfaces and binds tightly to extracellular matrix proteins including decorin (66). Perhaps intracellularly produced sPLA$_2$-IIA is directly channeled to glypican inside secretory vesicles prior to release to the extracellular compartment (Fig. 12). Further work is needed to understand why exogenously added sPLA$_2$-IIA is poorly active on mammalian cells, but a key factor is its poor ability to bind directly to the PC-rich outer layer of the plasma membrane (45).

The precise membrane compartment where glypican-shuttled sPLA$_2$s liberate AA for eicosanoid production in HEK293 cells remains to be established. The fact that sPLA$_2$-IIA binds extremely poorly to PC-rich vesicles and to the PC-rich outer face of the plasma membrane of mammalian cells but binds more than one million-fold tighter to anionic vesicles (43, 45) argues that glypican shuttling may bring this enzyme in contact with a membrane surface that is more enriched in acidic phospholipids than is the outer face of the plasma membrane. This is supported by the results obtained with sPLA$_2$-IIA and -V mutants, which retain high affinity for anionic phosphatidylglycerol vesicles but display altered affinity for PC vesicles (Fig. 7). Expression of sPLA$_2$-IIA in HEK293 cells leads to preferential AA release over oleic acid (37, 40) despite the fact that this enzyme shows virtually no *sn*-2 fatty acyl chain specificity *in vitro* (1, 67). This suggests that glypican shuttling brings the enzyme in contact with phospholipids that are en-

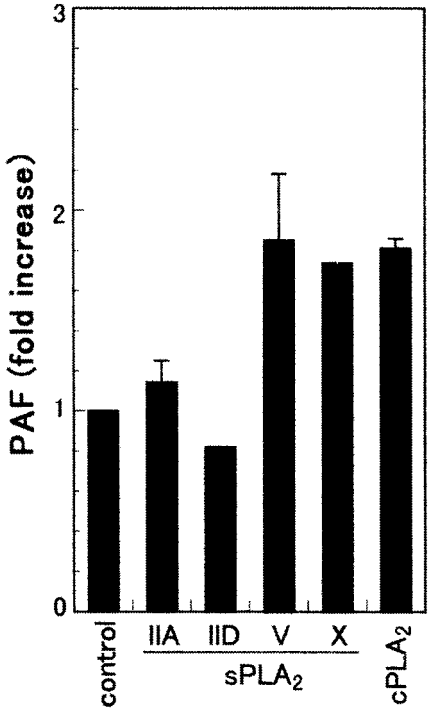

FIG. 10. **Effects of various PLA$_2$s on PAF generation in RBL-2H3 cells.** PAF generation by parental cells and cells expressing mouse sPLA$_2$-IIA, mouse sPLA$_2$-IID, rat sPLA$_2$-V, human sPLA$_2$-X, and mouse cPLA$_2$ (same transfectants as shown in Fig. 8) was quantified as described under "Experimental Procedures." Values are the means ± S.E. of three independent experiments.

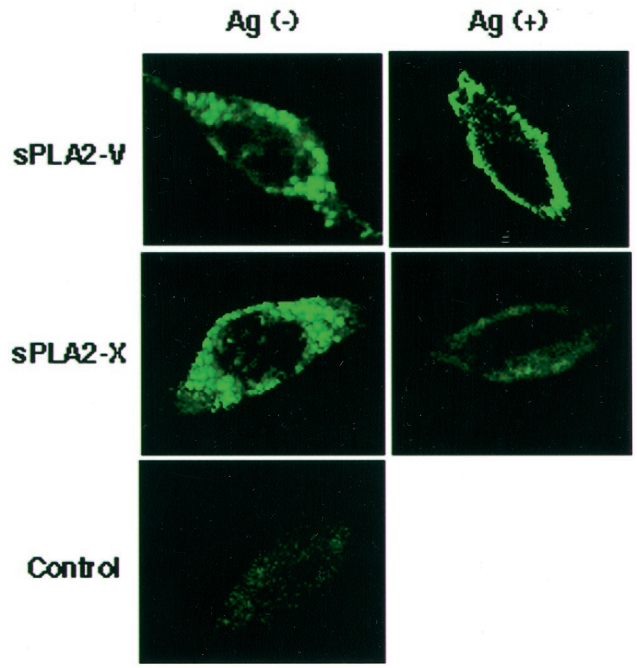

FIG. 11. **Immunocytostaining of sPLA$_2$-V and -X in RBL-2H3 transfectants.** Cells expressing FLAG-tagged rat sPLA$_2$-V and human sPLA$_2$-X before and 10 min after IgE/Ag activation were fixed, permeabilized, and then subjected to immunostaining using anti-FLAG and anti-sPLA$_2$-X antibodies, as detailed under "Experimental Procedures."

riched in AA. It is also noteworthy that AA release by the glypican-shuttled sPLA$_2$s occurs only in agonist-stimulated and not in unstimulated HEK293 cells. Perturbed membrane structures evoked by various cellular events, such as cPLA$_2$-directed membrane hydrolysis (37, 53, 68), lipid oxidation (68), and loss of lipid bilayer asymmetry (40), during cell activation may facilitate exposure of anionic phospholipids to these sPLA$_2$s in the sorted compartment. Finally, endogenous COX-2 induction in HEK293 cells is limited to glypican-binding sPLA$_2$s (Refs. 39 and 40 and Table I). The fact that significant IL-1-dependent PGE$_2$ production occurred in cells transfected with IIC-L95R/E102K alone argues that this mutant is also able to induce COX-2 expression.

The inability of the heparin-nonbinding sPLA$_2$-IIC, -IIE, and -IB to elicit AA release may be due to their poor affinity for heparan sulfate. Because these enzymes are mostly secreted into the culture medium, they could release AA from the outer surface of the plasma membrane. However, sPLA$_2$-IIC, -IIE, and -IB bind poorly to PC-rich vesicles and show very low activity when added exogenously to mammalian cells (45).[2] sPLA$_2$-X efficiently produces AA in transfected HEK293 cells by a mechanism that is distinct from that used by sPLA$_2$-IIA, -IID, and -V (Ref. 40 and Table I). Like sPLA$_2$-IIC and -IIE, this enzyme does not bind to heparanoids and accumulates in the culture medium. However, sPLA$_2$-X binds very efficiently to PC-rich membranes and is highly potent in releasing AA when added exogenously to a variety of mammalian cells (45). This and the fact that sPLA$_2$-X releases both oleic acid and AA (40) argues that this enzyme acts in a different HEK293 cell membrane compartment than does the heparanoid-binding sPLA$_2$s; it probably acts on the external face of the plasma membrane.

Lipid Interface-dependent Action—In contrast to the seemingly redundant functions of the three heparin-binding sPLA$_2$s (IIA, IID, and V) in AA release and PGE$_2$ generation in the HEK293 cell system, these enzymes display distinct roles in the regulation of immediate LTC$_4$ biosynthesis in rat mastocytoma RBL-2H3 cell transfectants. Among the sPLA$_2$s examined, only sPLA$_2$-V and -X, but not catalytic site mutants with poor enzymatic activity, exerted a potent enhancing effect on stimulus-dependent immediate production of LTC$_4$ (Figs. 8 and 9 and Table II). The same pattern was found for PGD$_2$ generation in these cells (18). These results are compatible with the previous observation that introduction of sPLA$_2$-V antisense DNA into MMC-34 mast cells reduced immediate PGD$_2$ generation (69). Failure of sPLA$_2$-IB, -IIA, -IIC, -IID, and -IIE to augment LTC$_4$ generation in activated mast cells implies that this event does not correlate with the heparanoid-binding tendency of sPLA$_2$s. Among these seven sPLA$_2$s, sPLA$_2$-V and -X are unique in being able to bind efficiently to PC-rich vesicles (44, 45). Thus, within the limits inherent in the method of forcible gene expression by transfection, the results suggest that in RBL-2H3 cells, which lack glypican, sPLA$_2$-V and -X could be acting on the PC-rich outer layer of the plasma membrane.

The plasma membrane target for sPLA$_2$-V and -X action in RBL-2H3 cells is further supported by immunocytochemical studies, which show that these two enzymes are associated with the plasma membrane after IgE/Ag activation (Fig. 11). sPLA$_2$-V provides a more intense signal than does sPLA$_2$-X on the plasma membrane of activated RBL-2H3 cells, suggesting that the former may not only bind directly to the PC-rich membrane, as does the latter, but also it may bind to specific proteoglycan species or other anionic components, which may be distributed uniformly on the external surface of the plasma membrane. The molecular entity of this putative cell surface component that may act as an adapter for sPLA$_2$-V on the plasma membrane of RBL-2H3 cells remains to be elucidated.

PC hydrolysis by sPLA$_2$-V and -X in activated mast cells is also supported by the observation that only these two sPLA$_2$s augment the production of PAF (Fig. 10), which is derived from 1-*O*-alkyl-PC. It is therefore likely that lyso-PAF produced by these PC-hydrolyzing sPLA$_2$s is supplied to lyso-PAF acetyl-

transferase for PAF production. These studies provide the first evidence that specific sPLA$_2$s can be coupled to 5-lipoxygenase and lyso-PAF acetyltransferase for the biosynthesis of LT and PAF, respectively. However, the possibility that this coupling occurred as a result of overexpression cannot be ruled out, and therefore this should be verified in a cell model with endogenous physiological levels of these enzymes in a future study.

Conclusions and Future Prospects—The gain-of-function studies reported here have revealed diverse aspects of the regulatory mechanisms for sPLA$_2$ function in two mammalian cell lines. Both heparan sulfate-dependent sorting into intracellular membrane compartments and interfacial binding to PC-rich membranes critically affect the mechanism of cellular action of sPLA$_2$s. Both of these parameters are influenced not only by the structural properties of each sPLA$_2$ but also by the presence of distinct secretory pathways in different cell types that are regulated by different proinflammatory stimuli. Remaining key questions include the generality of the two sPLA$_2$ regulatory mechanisms in other mammalian cell types, the role of sPLA$_2$ receptors in modulating the functions of a diverse set of sPLA$_2$s (11, 12), and the precise mechanism by which differential cell trafficking of sPLA$_2$s is coupled to differential *sn*-2 fatty acyl chain specificity for phospholipid hydrolysis, COX-2 induction, and interfacial binding of those sPLA$_2$s that cannot bind to PC-rich membranes. The fact that sPLA$_2$-IB, -IIC, and -IIE are not involved in AA release, at least in HEK293 and RBL-2H3 cells studied under the conditions reported here, suggest that these sPLA$_2$s may have novel functions which remain to be elucidated. Several studies have established that sPLA$_2$-IB from several animal species acts as a potent ligand for the M-type sPLA$_2$ receptor (11, 12).

Note Added in Proof—While this paper was under review, several important sPLA$_2$ papers related to this work have been reported. Cho and co-workers (72) have confirmed that exogenous human sPLA$_2$-V acts on human neutrophils through the heparanoid-independent, PC interface-dependent external plasma membrane mechanism. Moreover, they have shown that certain heparan sulfate proteoglycans on the neutrophil surface facilitates the internalization and subsequent proteolytic degradation of sPLA$_2$-V (72), the event also found by us for exogenous sPLA$_2$-IIA in mouse bone marrow-derived mast cells (73). Thus, in these cases, heparan sulfate proteoglycan works as a negative regulator of heparin-binding sPLA$_2$s. Hanasaki and his co-workers (74) have demonstrated that human sPLA$_2$-X releases AA from colon cancer cell lines through the external plasma membrane mechanism and proposed that this isozyme may be involved in COX-2-dependent exacerbation of colon tumorigenesis. Finally, the tenth member of mammalian sPLA$_2$, group XII, has been very recently identified (75), the functions of which remain to be elucidated.

REFERENCES

1. Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., and Kudo, I. (1997) *Crit. Rev. Immunol.* **17,** 225–283
2. Dennis, E. A. (1997) *Trends Biochem. Sci.* **22,** 1–2
3. Balsinde, J., and Dennis, E. A. (1997) *J. Biol. Chem.* **272,** 16069–16072
4. Tischfield, J. A. (1997) *J. Biol. Chem.* **272,** 17247–17250
5. Valentin, E., and Lambeau, G. (2000) *Biochim. Biophys. Acta*, **1488**, 59–70
6. Ishizaki, J., Suzuki, N., Higashino, K., Yokota, Y., Ono, T., Kawamoto, K., Fujii, N., Arita, H., and Hanasaki, K. (1999) *J. Biol. Chem.* **274,** 24973–24979
7. Valentin, E., Koduri, R. S., Scimeca, J. C., Carle, G., Gelb, M. H., Lazdunski, M, and Lambeau, G. (1999) *J. Biol. Chem.* **274,** 19152–19160
8. Valentin, E., Ghomashchi, F., Gelb, M. H., Lazdunski, M., and Lambeau, G. (1999) *J. Biol. Chem.* **274,** 31195–31202
9. Suzuki, N., Ishizaki, J., Yokota, Y., Higashino, K., Ono, T., Ikeda, M., Fujii, N., Kawamoto, K., and Hanasaki K. (2000) *J. Biol. Chem.* **275,** 5785–5793
10. Valentin, E., Ghomashchi, F., Gelb, M. H., Lazdunski, M., and Lambeau, G. (2000) *J. Biol. Chem.* **275,** 7492–7496
11. Lambeau, G., and Lazdunski, M. (1999) *Trends Pharmacol. Sci.* **20,** 162–170
12. Hanasaki, K., Yokota, Y., Ishizaki, J., Itoh, T., and Arita, H. (1997) *J. Biol. Chem.* **272,** 32792–32797
13. Wright, G. C., Weiss, J., Kim, K. S., Verheij, H., and Elsbach, P. (1990) *J. Clin. Invest.* **85,** 1925–1935
14. Laine, V. J., Grass, D. S., and Nevalainen, T. J. (1999) *J. Immunol.* **162,** 7402–7408
15. Laine, V. J., Grass, D. S., and Nevalainen, T. J. (2000) *Infect. Immun.* **68,** 87–92
16. MacPhee, M., Chepenik, K. P., Liddell, R., Nelson, K. K., Siracusa, L. D., and Buchberg, A. M. (1995) *Cell* **81,** 957–966
17. Matsuzawa, A., Murakami, M., Atsumi, G., Imai, K., Prados, P., Inoue, K., and Kudo, I. (1996) *Biochem. J.* **318,** 701–709
18. Enomoto, A., Murakami, M., Valentin, E., Lambeau, G., Gelb, M. H., and Kudo, I. (2000) *J. Immunol.* **165,** 4007–4014
19. Mounier, C., Franken, P. A., Verheij, H. M., and Bon, C. (1996) *Eur. J. Biochem.* **237,** 778–785
20. Ivandic, B., Castellani, L. W., Wang, X. P., Qiao, J. H., Mehrabian, M., Navab, M., Fogelman, A. M., Grass, D. S., Swanson, M. E., de Beer, M. C., de Beer, F., and Lusis A. J. (1999) *Arterioscler. Thromb. Vasc. Biol.* **19,** 1284–1290
21. Leitinger, N., Watson, A. D., Hama, S. Y., Ivandic, B., Qiao, J. H., Huber, J., Faull, K. F., Grass, D. S., Navab, M., Fogelman, A. M., de Beer, F. C., Lusis, A. J., and Berliner, J. A. (1999) *Arterioscler. Thromb. Vasc. Biol.* **19,** 1291–1298
22. Verheij, H. M., Slotboom, A. J., and De Haas, G. (1981) *Rev. Physiol. Biochem. Pharmacol.* **91,** 91–203
23. Nakazato, Y., Simonson, M. S., Herman, W. H., Konieczkowski, M., and Sedor, J. R. (1991) *J. Biol. Chem.* **266,** 14119–14127
24. Kuwata, H., Nakatani, Y., Murakami, M., and Kudo, I. (1998) *J. Biol. Chem.* **273,** 1733–1740
25. Pfeilschifter, J., Schalkwijk, C., Briner, V. A., and van den Bosch, H. (1993) *J. Clin. Invest.* **92,** 2516–2523
26. Nakano, T., Ohara, O., Teraoka, H., and Arita, H. (1990) *J. Biol. Chem.* **265,** 12745–12748
27. Chen, J., Engle, S. J., Seilhamer, J. J., and Tischfield, J. A. (1994) *J. Biol. Chem.* **269,** 23018–23024
28. Chen, J., Engle, S. J., Seilhamer, J. J., and Tischfield, J. A. (1994) *J. Biol. Chem.* **269,** 2365–2368
29. Sawada, H., Murakami, M., Enomoto, A., Shimbara, S., and Kudo, I. (1999) *Eur. J. Biochem.* **263,** 826–835
30. Shinohara, H., Balboa, M. A., Johnson, C. A., Balsinde, J., and Dennis, E. A. (1999) *J. Biol. Chem.* **274,** 12263–12268
31. Balsinde, J., Shinohara, H., Lefkowitz, L. J., Johnson, C. A., Balboa, M. A., and Dennis, E. A. (1999) *J. Biol. Chem.* **274,** 25967–25970
32. Cupillard, L., Koumanov, K., Mattei, M.-G., Ladzdunski, M., and Lambeau, G. (1997) *J. Biol. Chem.* **272,** 15145–15152
33. Murakami, M., Kudo, I., and Inoue, K. (1993) *J. Biol. Chem.* **268,** 839–844
34. Suga, H., Murakami, M., Kudo, I., and Inoue, K. (1993) *Eur. J. Biochem.* **218,** 807–813
35. Murakami, M., Nakatani, Y., and Kudo, I. (1996) *J. Biol. Chem.* **271,** 30041–30051
36. Tada, K., Murakami, M., Kambe, T., and Kudo, I. (1998) *J. Immunol.* **161,** 5008–5015
37. Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M. V., Tischfield, J. A., and Kudo, I. (1998) *J. Biol. Chem.* **273,** 14411–14423
38. Murakami, M., Kambe, T., Shimbara, S., and Kudo, I. (1999) *J. Biol. Chem.* **274,** 3103–3115
39. Murakami, M., Kambe, T., Shimbara, S., Yamamoto, S., Kuwata, H., and Kudo, I. (1999) *J. Biol. Chem.* **274,** 29927–29936
40. Murakami, M., Kambe, T., Shimbara, S., Higashino, K., Hanasaki, K., Arita, H., Horiguchi, M., Arita, M., Arai, H., Inoue, K., and Kudo, I. (1999) *J. Biol. Chem.* **274,** 31435–31444
41. Murakami, M., Kudo, I., and Inoue, K. (1991) *FEBS Lett.* **294,** 247–251
42. Kudo, I., Murakami, M., Hara, S., and Inoue, K. (1993) *Biochim. Biophys. Acta* **1107,** 217–231
43. Koduri, R. S., Baker, S. F., Snitko, Y., Han, S.-K., Cho, W., Wilton, D. C., and Gelb, M. H. (1998) *J. Biol. Chem.* **273,** 32142–32153
44. Han, S. K., Kim, K. P., Koduri, R., Bittova, L., Munoz, N. M., Leff, A. R., Wilton, W., Gelb, M. H., and Cho, W. (1999) *J. Biol. Chem.* **274,** 11881–11888
45. Bezzine, S., Koduri, R. S., Valentin, E., Murakami, M., Kudo, I., Ghomashchi, F., Sadilek, M., Lambeau, G., and Gelb, M. H. (2000) *J. Biol. Chem.* **275,** 3179–3191
46. Hanasaki, K., Ono, T., Saiga, A., Morita, Y., Ikeda, M., Kawamoto, K., Higashino, K., Nakano, K., Yamada, K., Ishizaki, J., and Arita, H. (1999) *J. Biol. Chem.* **274,** 34203–34211
47. Baker, S. F., Othman,, R., and Wilton, D. C. (1998) *Biochemistry* **37,** 13203–13211
48. Dole, V. P., and Meinertz, H. (1960) *J. Biol. Chem.* **235,** 2595–2599
49. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–918
50. Nakajima, K., Murakami, M., Yanoshita, R., Samejima, Y., Karasawa, K., Setaka, M., Nojima, S., and Kudo, I. (1997) *J. Biol. Chem.* **272,** 19708–19713
51. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) *Nature* **390,** 618–621
52. Nakatani, Y., Tanioka, Y., Sunaga, S., Murakami, M., and Kudo, I. (2000) *J. Biol. Chem.* **275,** 1161–1168
53. Atsumi G., Murakami, M., Kojima, K., Hadano, A., Tajima,, M., and Kudo, I. (2000) *J. Biol. Chem.* **275,** 18248–18258
54. Anderson, R. G. W. (1998) *Annu. Rev. Biochem.* **67,** 199–225
55. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem.* **273,** 5419–5422
56. Brown, D. A., and London, E. (2000) *J. Biol. Chem.* **275,** 17221–17224
57. Fujishima, H., Sanchez Mejia, R. O., Bingham, C. O., Lam, B. K., Sapirstein, A., Bonventre, J. V., Austen, K. F., and Arm, J. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4803–4808
58. Kurihara, H., Nakano, T., Takasu, N., and Arita, H. (1991) *Biochim. Biophys. Acta* **1082,** 285–292
59. Couturier, C., Brouillet, A., Couriaud, C., Koumanov, K., Bereziat, G., and Andreani, M. (1999) *J. Biol. Chem.* **274,** 23085–23093
60. Murakami, M., Kudo, I., Suwa, Y., and Inoue, K. (1992) *Eur. J. Biochem.* **209,** 257–265

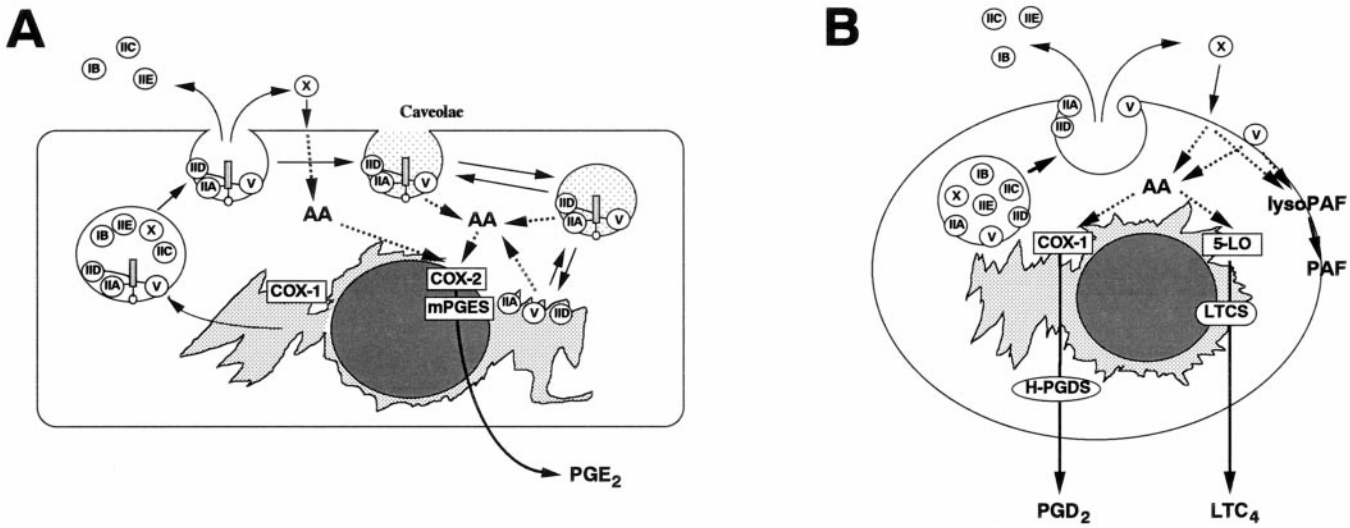

FIG. 12. **Two sPLA$_2$-dependent eicosanoid-biosynthetic pathways.** *A*, the glypican-shuttling mechanism. In fibroblasts and several other adherent cells, intracellularly produced heparin-binding sPLA$_2$s (IIA, IID, and V) may be directly channeled to heparan sulfate chains of glypican inside secretory vesicles and prior to release to the extracellular space. Glypican-bound sPLA$_2$s are then delivered into caveolae signalsomes, which shuttle between the plasma membrane and intracellular membrane compartments through potocytosis (54). Glypican-bound sPLA$_2$s release AA selectively from these compartmentalized membrane microdomains (37, 39) that may be enriched in anionic phospholipids. sPLA$_2$-X, which does not enter the glypican-shuttling route, is released into the extracellular medium and acts on the PC-rich outer surface of the plasma membrane to release AA and other fatty acids (40). AA released by these sPLA$_2$s is supplied to the delayed PGE$_2$-biosynthetic route that involves the two inducible perinuclear enzymes, COX-2 and membrane-bound PGE$_2$ synthase (*mPGES*) (64, 65). Preferential coupling of sPLA$_2$s with COX-2 to COX-1 may result from the fact that COX-2 is favored over COX-1 when the AA supply is limited (38) and that mPGES is preferentially coupled with COX-2 (65). sPLA$_2$-IB, IIC, and IIE, which show low interfacial binding to PC-rich membranes and do not bind glypican, do not release AA in this setting. *B*, the glypican-independent, plasma membrane mechanism. In mast cells and probably other hematopoietic cells in which caveolae are poorly developed and glypican is poorly expressed, if at all, sPLA$_2$s are stored in secretory granules and undergo rapid exocytosis after cell activation. sPLA$_2$s with high heparin affinity (IIA and IID) are associated with membranous sites where fusion between plasma membrane and granule membrane occurs and contribute to local production of lysophospholipids, which further facilitates membrane fusion leading to enhanced degranulation (18). sPLA$_2$-V, which shows intermediate heparin affinity, is distributed uniformly on the plasma membrane surface. These distributions of sPLA$_2$-IIA, -IID, and -V may be mediated by different sets of heparan sulfate proteoglycans or other anionic components. sPLA$_2$-X, -IB, -IIC, and -IIE are released into the extracellular medium. Only the two PC-hydrolyzing enzymes, sPLA$_2$-V and -X, are capable of releasing AA from the outer plasma membrane. The AA released is supplied sequentially to constitutive COX-1 and hematopoietic PGD$_2$ synthase (*H-PGDS*) for immediate production of PGD$_2$ and to 5-lipoxygenase and LTC$_4$ synthase (*LTCS*) for immediate production of LTC$_4$ (70, 71). This action of sPLA$_2$-V and -X is similar to that seen when these enzymes are added exogenously (44–46). sPLA$_2$-V and -X also have the ability to augment PAF biosynthesis through generation of lysoPAF.

61. Horigome, K., Hayakawa, M., Inoue, K., and Nojima, S. (1987) *J. Biochem. (Tokyo)* **101,** 625–631
62. Wright, G. W., Ooi, C. E., Weiss, J., and Elsbach, P. (1990) *J. Biol. Chem.* **265,** 6675–6681
63. Dua, R., and Cho, W. (1994) *Eur. J. Biochem.* **221,** 481–490
64. Spencer, A. G., Woods, J. W., Arakawa, T., Singler, I. I., and Smith, W. L. (1998) *J. Biol. Chem.* **273,** 9886–9893
65. Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh-ishi, S., and Kudo, I. (2000) *J. Biol. Chem.* **275,** 82783–82792
66. Sartipy, P., Johansen, B., Gasvik, K., and Hurt-Camejo, E. (2000) *Circ. Res.* **86,** 707–714
67. Bayburt, T., Yu, B. Z., Lin, H. K., Browning, J., Jain, M. K., and Gelb, M. H. (1993) *Biochemistry* **32,** 573–582
68. Kuwata, H., Yamamoto, S., Miyazaki, Y., Shimbara, S., Nakatani, Y., Suzuki, H., Ueda, N., Yamamoto, S., Murakami, M., and Kudo, I. (2000) *J. Immunol.* **165,** 4024–4031
69. Reddy, S. T., Winstead, M. V., Tischfield, J. A., and Herschman, H. R. (1997) *J. Biol. Chem.* **272,** 13591–13596
70. Murakami, M., Matsumoto, R., Urade, Y., Austen, K. F., and Arm, J. P. (1995) *J. Biol. Chem.* **270,** 3239–3246
71. Murakami, M., Austen, K. F., Bingham III, C. O., Friend, D. S., Penrose, J. F., and Arm, J. P. (1995) *J. Biol. Chem.* **270,** 22653–22656
72. Kim, K.-P., Rafter, J. D. Bittova, L., Han, S.-K., Snitko, Y., Munoz, N. M., Leff, A. R., and Cho, W. (December 15, 2000) *J. Biol. Chem.* 10.1074/jbc.M004604200
73. Enomoto, A., Murakami, M., and Kudo, I. (2000) *Biochem. Biophys. Res. Commun.* **276,** 667–672
74. Morioka, Y., Ikeda, M., Saiga, A., Fujii, N., Ishimoto, Y., Arita, H., and Hanasaki, K. (2000) *FEBS Lett.* **387,** 262–266
75. Gelb, M. H., Valentin, E., Ghomashchi, F., Lazdunski, M., and Lambeau, G. (2000) *J. Biol. Chem.* **275,** 39823–39826